Annual Meeting

Multistate Project NE1227: Ovarian Influences on Reproductive Success in Ruminants

University of New Hampshire, Durham NH May 18-20, 2015

AGENDA

Monday, May 18, 2015

1. Check into hotel (Holiday Inn Express, Durham NH, contact: Dave Townson, dave.townson@unh.edu or Paul Tsang, paul.tsang@unh.edu with any questions.)
2. Dinner @ restaurant in Three Chimneys Inn (Paul will be sending information)

Tuesday, May 19, 2015 –Breakfast on own; light snacks, coffee/tea available at meeting

8:00 AM.

1. Meet in Conference Room at UNH (Dave/Paul will be providing campus maps). Welcome and introductions at 8:00AM.
2. Station reports for NE-1227 begin 8:15A

Presentation of Station Reports:

PA – Pate – Luteal Exosomes & Other Studies

PA – Ott

NH – Townson/Tsang

NY - Fortune

NY – Quirk

NY – Butler

Discussion of rewrite of Regional Project – We do not need to complete the rewrite this year but we need to make our plans for next year.

3. Lunch –boxed lunches
4. Reconvene for station reports at 1:30 PM

Continuing Presentation of Station Reports:

NE – Jen Wood

WV – Inskeep/Yao/Flores

MA – Fissore

KY - Bridges

MS – Memili

WI - Wiltbank

Others?

Further Discussion of potential projects and new research in new regional project.

5. Finish by 4:00 to leave for Portsmouth by 4:30 PM
6. Dinner in Portsmouth at SURF

Wednesday, May 20, 2015–Breakfast on own

8:30 AM.

1. Finish presentation of station reports
2. NIFA information – Adele Turzillo
3. Administrator comments – Gary Thompson
4. Further Discussion of rewrite of NE Regional Project

   Elect new director; identify meeting time and place for 2016 meeting, Depart by 12:00 Noon

WEST VIRGINIA AGRICULTURAL AND FORESTRY EXPERIMENT STATION
ANNUAL REPORT – 2015

1. Project: NE-1227, Hatch 476. Ovarian Influences on Reproductive Success in Ruminants

2. ***Participants*** **(FTE)** Cooperating Agencies and Principal Leaders:
Division of Animal and Nutritional Sciences - E. K. Inskeep (0.2), R. A. Dailey (0.1), J. Yao (0.3), L. Wang (0.5), J. Hand (0.5) M. Graham (0.5), K. Akers (0.0).
Department of Biology – J.A. Flores (0.2), M. Wright (0.1), E. Bowdridge (0.5)

3. ***Target Audience***

Target Audiences: Scientists working in complementary areas, producers, graduate students, technicians, undergraduate students, and veterinary medicine students.

Efforts: conference and meeting presentations, peer-reviewed manuscripts, formal classroom instruction, laboratory instruction, development of curriculum and case study scenarios, experiential learning opportunities.

4. ***Products***

Wang L and Yao J. 2014. The microRNAs important for ovarian and early embryonic development in cattle. Turkish Journal of Veterinary and Animal Sciences. 38: 599-605. doi:10.3906/vet-1409-1.(review).

Gupta PSP, Folger JK, Rajput S, Lv L, Yao J, Ireland J and Smith GW. 2014. Regulation and regulatory role of WNT signaling in potentiating FSH action during bovine dominant follicle selection. PLoS ONE 9(6): e100201. doi:10.1371/journal.pone.0100201.

Bowdridge, E.C., Goravanahally M.P., Inskeep, E.K., Flores, J.A. 2015. Activation of adenosine monophosphate activated protein kinase is an additional mechanism that participates in mediating inhibitory actions of prostaglandin f2 alpha in mature, but not developing, bovine corpora lutea. Biol. Reprod. (Accepted)

Bowdridge, E.C., Inskeep, E.K., Flores, J.A. 2015.The role of adenosine monophosphate activated kinase in luteal progesterone production. 48th Annual Meeting Society for the Study of Reproduction, San Juan, Puerto Rico. June 18 – 22, 2015.

Bowdridge, E.C. 2014. The role of adenosine monophosphate activated kinase in luteal progesterone production. Doctoral dissertation,
West Virginia University. 95 pages.

Graham Megan Renee 2014. Effects of lipopolysaccharide induced inflammatory response on early embryo survival in ewes. M.S. Thesis, West Virginia University. 84 pages.

Akers Keli Ann. 2014. Changes in body condition from prepartum to postpartum on health and reproduction in dairy cattle. M.S. Thesis, West Virginia University

Goodman, R. L. and E. K. Inskeep. 2015. Control of the Ovarian Cycle of the Sheep. Chapter 27 in Knobil and Neill's Physiology of Reproduction. Fourth Edition. Edited by Tony Plant and Tony Zeleznik. Academic Press/Elsevier, London. Volume 2. 1259-1305. Cited last year as in press.

Holler, Tammy L., Matthew Dean, Todd Taylor, Daniel H. Poole, M. L. Thonney, D. L. Thomas, J. L. Pate, N. Whitley, R. A. Dailey, and E. K. Inskeep. 2014. Effects of service sire on prenatal mortality and prolificacy in ewes. J. Anim. Sci. 92:3108-3115. Cited last year as in press.

5. Progress of Work and Principal Accomplishments:

Objective 1. Identify intracellular signaling pathways and gene expression regulatory mechanisms within the ovary, embryo, or female reproductive tract that promote oocyte growth and maturation, fertilization, early embryonic development, and establishment and maintenance of pregnancy.

A. Discovery of a Novel Oocyte-Specific KRAB-Containing Zinc Finger Protein Required for Early Embryogenesis in Cattle. Jacqelyn M. Hand[1], Kun Zhang[2], Lei Wang[1], George W. Smith[2] and Jianbo Yao[1]
[1]West Virginia University, Division of Animal and Nutritional Sciences, Morgantown, WV, 26505
[2]Michigan State University, Department of Animal Science, East Lansing, MI, 48824

Zinc finger (ZNF) transcription factors are known to interact with DNA through zinc finger motifs and play important roles in a variety of cellular functions, including cell growth, proliferation, development, apoptosis, and intracellular signal transduction. One-third of ZNF proteins contain a highly conserved N-terminal motif known as the KRAB domain, which acts as a potent, DNA-binding dependent transcriptional repression module. To date, ZNF proteins expressed specifically in mammalian oocytes have not been reported. RNA sequencing of a bovine oocyte library uncovered a highly abundant transcript that matches an uncharacterized gene in the NCBI database. Through cDNA cloning of the novel gene (*ZNFO*) a transcript containing a 2,145 bp open reading frame that codes for a protein of 714 amino acids with a conserved KRAB domain at the N-terminus and nine zinc finger motifs at the C-terminus was identified. *ZNFO* mRNA is readily detectable in fetal ovaries and was undetectable by RT-PCR in somatic tissues including granulosa and theca cells. Real-time PCR analysis revealed *ZNFO* mRNA is highly abundant in GV and MII stage oocytes as well as in pronuclear to 8-cell stage embryos but undetectable in blastocyst stage embryos (n = 4 pools of 10 embryos/stage; $P < 0.05$). Immunohistochemical analysis detected ZNFO protein in oocytes throughout folliculogenesis. Based on the well-conserved functions of KRAB-containing ZNF transcription factors and the current spatial and temporal observations of ZNFO, our working hypothesis is

that ZNFO functions as a transcriptional regulator required during early embryonic developmental events and mediates downstream activity of potential targets through a cis-acting ZNFO consensus recognition sequence. To begin to test this hypothesis, zygotes were generated by *in vitro* maturation and fertilization of oocytes, and injected with small interfering RNA (siRNA) designed to knockdown *ZNFO*. Cleavage rates were not affected by ZNFO siRNA injection ($P > 0.05$). However, embryonic development to 8- to 16-cell stage and blastocyst stage was significantly reduced relative to the uninjected and negative control siRNA-injected embryos (n = 3 replicates; 25-30 embryos/treatment; $P < 0.05$). Next, to investigate the DNA-binding properties of ZNFO, cyclic amplification and selection of targets (CASTing) analysis was performed using a double-stranded oligonucleotide library containing a random core that was incubated with GST-fused ZNFO protein for seven rounds of high affinity selection. The products were sequenced on an Illumina platform. Thirteen cis-acting sites were identified as potential ZNFO DNA binding elements (ZBEs). The ZBEs represent consensus sequences recognized by ZNFO in order to bind and regulate potential target transcripts. In addition, transfection studies verified that a ZNFO-GFP fusion protein localizes specifically to the nucleus, further supporting proposed function in transcriptional regulation. Results of described studies demonstrate that ZNFO is a maternally-derived oocyte-specific factor required for early embryonic development in cattle, and possesses DNA-binding ability, possibly by identified consensus sequences. (Supported by Agriculture and Food Research Initiative Competitive Grant no. 2009-65203-05706 from the USDA National Institute of Food and Agriculture).

B. The role of adenosine monophosphate activated kinase in luteal progesterone production. Bowdridge, E.C., Inskeep, E.K., Flores, J.A.

The luteolytic actions of $PGF_{2\alpha}$ are thought to be mediated through an elevation of cytosolic Ca^{2+} and subsequently activation of protein kinase C (PKC). Our laboratory has examined the possibility that elevation of cytosolic Ca^{2+} activates an additional intracellular effector, namely, calcium/calmodulin-dependent kinase kinase 2 (*CAMKK2*). Expression of both CAMKK2 and its potential phosphorylation target, adenosine monophosphate activated protein kinase (AMPK) increased as the CL developed to a mature stage. Furthermore, activation of the $PGF_{2\alpha}$ receptor (FP) induced rapid phosphorylation of AMPK, which was blocked by a CAMKK2 inhibitor. In mature CL, the effect of $PGF_{2\alpha}$ on basal progesterone (P4) production was eliminated by

addition of an AMPK-specific inhibitor, dorsomorphin dichloride (DM). Therefore, the objectives of this study were to explore alternative downstream components activated by the rise in cytosolic [Ca^{2+}], such as AMPK, in developing and mature bovine CL and identify potential distal targets of AMPK, such as cholesterol transport mechanisms, when the FP receptor is activated during functional regression. In experiment 1, changes in basal P4 secretion *in vitro* were determined in response AMPK activation via metformin (met) or 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) in developing (day 4) and mature (day 10) bovine CL. On d 4 or d 10 (d 4; n = 5; d 10; n = 5) CL were collected via supravaginal incision under epidural anesthesia. Luteal slices were incubated for 2-h at 37°C, shaking at 200 rpm with MEM (control), $PGF_{2\alpha}$ (1.0 μg/ml), met (10 mM), or AICAR (7.5 mM). Production of P4 in d 4 CL was not significantly affected by met or AICAR. However, P4 production in d 10 CL decreased with either met (0.39 ± 0.20; $P = 0.006$) or AICAR (0.40 ± 0.15; $P = 0.0117$) compared to control (0.99 ± 0.32). Direct activation of AMPK in mature CL resulted in decreased basal P4 production, similar to activation by $PGF_{2\alpha}$. Therefore, potential distal targets of AMPK during induced functional regression via exogenous $PGF_{2\alpha}$ were examined in the mature CL. Specifically, changes in protein expression of AMPK, phosphorylated AMPK (P-AMPK), steroidogenic acute regulatory (StAR), and cholesterol transport proteins (LDL, SRB-1, ACAT-1) were examined in experiment 2. Day 10 CL were removed supravaginally at 0 (n = 5), 2 (n = 5), or 4 (n = 5) hours after s.c. injection of $PGF_{2\alpha}$ or saline. Serum and luteal P4 concentration and content, respectively, decreased at 2 h (2.08 ng/ml vs. 2.96 ng/ml; $P = 0.054$) and 4 h (1.57 ng/ml vs. 2.96 ng/ml; $P = 0.013$) after $PGF_{2\alpha}$ administration (d10-h0 = 5.05 ng/ml; d10-h2 = 2.03 ng/ml; d10-h4 = 1.74 ng/ml) Protein expression of LDL decreased at 2 h (0.79 vs. 0.41; $P = 0.09$) and 4 h after $PGF_{2\alpha}$ injection (0.79 vs. 0.13; $P = 0.004$). Protein expression of ACAT-1 (0.67 vs. 0.21, $P = 0.010$) and StAR (3.62 vs. 1.25, $P = 0.01$) increased 4 h after $PGF_{2\alpha}$. No difference in SRB-1, AMPK, or P-AMPK protein expression occurred 2 or 4 h after $PGF_{2\alpha}$. During induced functional regression, alterations in LDL and additional cholesterol transport proteins, accounted, at least in part for the decrease in luteal and serum P4 concentrations. In conclusion, developmental differences in signal transduction associated with FP, specifically CAMKK2 and AMPK , contribute to differences in the ability of $PGF_{2\alpha}$ to induce functional luteal regression in mature, but not developing, bovine CL. Furthermore, cholesterol transport via LDL might be the target of this luteal developmental difference.

C. Investigations of early embryonic loss via gram-negative bacterial infections, such as mastitis. Jessalyn Hadfield and Robert Dailey

Early pregnant ewes (d 5 or 6 post coitus) are treated with the gram-negative bacteria cell wall component, lipopolysaccharide (LPS) to induce the inflammatory and acute phase response by the innate immune system that occurs during bacterial infections. The current aim is to determine if feeding polyunsaturated fatty acids (PUFAs) has a beneficial effect on the innate immune response to LPS. Such an effect might reduce embryonic loss in cases of bacterial infection. The allogenic fetus was once thought to be ignored by the maternal immune system, but the immune system plays a critical role in the establishment, maintenance, and termination of pregnancy and there is a delicate balance between tolerance and rejection of a fetus. A bacterial infection early in gestation can tip that balance towards rejection as the dam tries to clear the infection and save herself over a pregnancy that is not yet well established.

Several types of immune cells and factors that are critical for implantation success and maintenance of pregnancy. The production of regulatory T cells (Tregs) is induced by transforming growth factor β (TGF-β) in the seminal plasma and these cells are in charge of maintaining a suppressive uterine environment where paternal antigens are expressed. Uterine natural killer cells (uterine NKs) also are essential for establishment of pregnancy, specifically in angiogenesis and transformation of the uterine spiral arteries. These NKs have all the normal killing mechanisms of peripheral NKs, however they do not act like peripheral NKs, unless there is a stimulus that instruct them to do so. Immune cells are plastic, in that they can change function based on the environment and cytokines being produced by other cells. In response to pro-inflammatory cytokines, Tregs can lose their suppressive functions resulting in loss of fetal tolerance. Uterine NKs can actually turn on the fetus and start killing fetal cells in response to an inflammatory environment. For successful implantation, the anti-inflammatory interleukin-10 is critical. An infection early in pregnancy causing an overabundance of pro-inflammatory cytokines in the uterus can result in implantation failure or later pregnancy complications. Therefore, if the inflammatory response that occurs during a bacterial infection can be reduced, the amount of embryonic loss may also be reduced.

PUFAs have been shown to have anti-inflammatory effects and be beneficial for several conditions. To evaluate effects of omega-3 fatty acids, whole flaxseed is being fed to ewes

starting shortly before breeding through the time of implantation. Flaxseed is high in alpha-linolenic acid which can be converted into the longer chain omega-3 FAs in the body. Feeding diets high in omega-3/omega-6 have increased pregnancy per artificial insemination and reduced pregnancy loss. In response to an LPS challenge, animals fed PUFAs had reduced pro-inflammatory cytokines. PUFAs take the place of arachidonic acid in plasma membranes limiting the substrate for cyclooxygenase & reducing the production of the 2 series prostaglandins. Instead, there is a shift towards production of the 3 series prostaglandins with diets high in omega-3s. In addition, PUFAs can reduce transcription of genes for the pro-inflammatory cytokines. The current experiments have four groups: (1) ewes fed flaxseed & given LPS; (2. ewes fed flaxseed & are given PBS; (3) ewes fed a control diet & given LPS; and (4) ewes fed a control diet & given PBS. Treatments occur on d 5-6 post-coitus and blood samples are taken for the following 12 hours. Measures will include the number of white blood cells, protein and mRNA of pro-inflammatory cytokines (interleukin-1β, TGF-α, interleukin-6), the acute phase proteins serum amyloid A & haptoglobin (controlled by pro-inflammatory cytokines and the hypothalamic-pituitary-adrenal axis), and the complement components C3a and C5a. On day 16, when implantation should start, both IL-10 and Tregs, which are critical for successful implantation, will be measured. Progesterone will be measured on d9/10 and d25/26 to monitor pregnancy and placental efficiency (grams of fetus per gram of placenta) will be determined at parturition. One LPS challenge has been completed except for collection of the samples at the later time points in gestation. The experiment will be repeated in fall, 2015.

D. Effects of lipopolysaccharide induced inflammatory response on early embryo survival in ewes. Megan Graham and Robert Dailey

Early pregnant ewes, 5 or 6 day post coitus (dpc) were used as a model to study early embryonic loss via gram-negative bacterial infections, such as mastitis. Ewes 5/6 dpc were injected with a gram-negative bacteria cell wall component, lipopolysaccharide (LPS) or tumor necrosis factor-alpha (TNF-α), to induce an innate immune system acute phase response (APR). The induction of the APR and its reactant molecules, such as TNF-α and acute phase proteins (APP), haptoglobin (Hp) and serum amyloid A (SAA), was initiated to study their effects on embryonic loss. In addition, ewes were injected with LPS plus dexamethasone (dex) to study its effects on indirectly altering embryonic loss through toll-like receptor 4 (TLR4). Thirty-eight

Dorset x Texel ewes were synchronized for estrus and bred by fertile rams. On 5 or 6 dpc, ewes were assigned to one of four treatment groups per pen and received via the jugular vein either 2.5 mL of 0.1% BSA/PBS (controls, n=9), 2.5 mL of 2.5 μg/kg of LPS (n=9), 5 mL of 1 μg/kg of TNF-α (n=10) in two bolus injections given thirty minutes apart, or 2.5 mL of solution containing 2.5μg/kg of LPS after having received 3.5 mL of solution containing 0.14 mg/kg BW im dexamethasone (DEX, n=10) at -12 and 0 hours. Plasma was collected from the jugular vein before challenge, followed by post challenge samples every 30 minutes until 3 hours and every hour until 12 hours, and once at 24, 36, and 48 hours. In addition, behavioral changes and rectal temperature were documented before challenge injections followed every hour for 12 hours post challenge and processed soon after collection for total white blood cell counts, and plasma was stored at -80°C. A white blood cell differentiation was determined by staining and counting one hundred cells classified among monocyte, lymphocyte, eosinophil, neutrophil, or basophil cell types. Assays were conducted for APR reactants, TNF-α, SAA, Hp. Jugular samples were collected in EDTA treated tubes on days 9 or 10 and 25 or 26 pcs for determination of concentrations of progesterone (P4) for evaluation of luteal function. At day 25 or 26 pcs, detection for pregnancy was examined. Intoxication of day 5 or 6 pregnant ewes with LPS, TNF-α, or LPS+Dex did not differ in pregnancy status among treatment groups ($p=0.298$). Total white blood cell count differed by treatment ($p<0.0001$), hour ($p<0.001$), and treatment by hour ($p<0.0001$). There was an effect of treatment on lymphocytes ($p<0.0001$) and monocytes ($p=0.0103$). There was an effect of hour on lymphocytes ($p<0.0001$), neutrophils ($p<0.0001$), and monocytes ($p<0.0001$). There was an effect of treatment by hour on neutrophils ($p=0.0047$). There was no difference in Hp concentration by treatment ($p=0.0859$), hour ($p=0.4317$), and treatment by hour ($p=0.0996$). There was a difference in SAA concentration by treatment ($p<0.001$), hour ($p<0.001$), and treatment by hour ($p<0.001$). The APR was elicited via treatment with LPS. Although LPS affected pregnancy, treatment with TNF-α did not. Dexamethasone attenuated the inflammatory response but did not increase pregnancy outcome.

E. Changes in body condition from prepartum to postpartum on health and reproduction in dairy cattle. Keli Ann Akers and Robert Dailey

The aims of this study were to body condition score(BCS), 72 Holstein-Friesian dairy cows at prepartum,; compare lean, moderate, and over-conditioned categories; and relate any changes associated with the various time points with reproductive success by measuring BCS,

hematocrit, total white blood cell (WBC) differential, haptoglobin (Hp) concentrations, milk yield, and monitoring health status. The body condition score changed significantly with time with the greatest unit loss being in the over-conditioned cattle from d -30 to d 35 postpartum. The BCS losses for each category did not have a significant effect on reproductive success at any time. Health disorders were significantly related to parity. The health status at d 0 to 10 and during pre-breeding (d 20 to 26) postpartum had a significant effect on reproductive success. The hematocrit values were significantly reduced at d 20 to 26 postpartum. Pregnancy success was associated with Hp and milk yield with higher Hp concentrations and higher milk yield having the highest pregnancy rate. The white blood cell differential count was significantly different at certain sampling time points for monocytes, total neutrophils, and eosinophils; with the highest concentration for each at parturition. Lymphocyte concentrations did not differ. The study revealed that body condition, hematocrit, and parity were not good indicators of reproductive success. Parity of an animal had no effect on reproductive success but its association with prevalence of disease and health disorders in early postpartum decreased pregnancy success to first artificial insemination. Likelihood of successful pregnancy was high if both Hp and milk yield were high. The WBC counts portrayed lower than normal concentrations of lymphocytes and elevated levels of total neutrophils.

F. Cellular biomarkers to predict fertility in dairy cattle. Melissa Wise and Robert Dailey

A persistent challenge of dairy herd management is maintaining an efficient calving interval. By minimizing services per conception, producers can save considerable time and money spent on getting cows pregnant. Fertile cows that breed back efficiently translate into economical calving intervals and decreased costs associated with heat detection and breeding. Thus, optimizing fertility is financially important to maintain an economically viable dairy operation.

Physical demands of calving and lactation cause partitioning of nutrients towards increased milk production, resulting in a negative energy balance of the cow after calving. Suppression of immunity commonly occurs during this transition, presumably resulting from physical and hormonal stress. Outcomes of immunosuppression during the transition phase may include an increased incidence of uterine infection and mastitis. These changes impact cow health and increase time to next conception (Barker et al., 1998). Cows that were sick at their

pre-breeding health examination (20-26 days in milk (DIM)) had extended intervals to rebreeding (Akers, 2014). Cows with mastitis have increased somatic cell counts, inflammation, and acute phase responses in the udder that are reflected in a systemic innate immune response (Bochsler and Slauson, 2002). In addition to cellular innate immune responses, the host elicits an acute phase response, which may occur prior to and concurrently with active inflammation. Acute phase proteins, such as haptoglobin, affect target cells by changing metabolic, biochemical, and hormonal cell functions with the goal of restoring homeostasis (Gruys et al., 2005). Haptoglobin concentrations have been utilized as a means of identifying sick cows (Skinner et al., 1991; Alsemgeest et al., 1994; Sheldon et al., 2001).

In contrast, previous research has shown that cows with low incidence of disease at 20-26 DIM that also had high systemic haptoglobin (> 3 mg/ml), above herd average milk yield (42.67 kg/day), and low blood lymphocyte (< 2.5 cells/μL) and monocyte (< 0.8 cells/μL) counts had higher pregnancy rates to first service than their herd mates. Lymphocyte and monocyte concentrations were correlated with total white blood cells in circulation (Akers, 2014).

The specific aim of this project is to associate leukocyte counts, concentrations of haptoglobin, and milk yield on reproductive success in dairy cattle. Leukocyte counts will be compared systemically, in quarter milk samples, and the uterine endothelium. Haptoglobin will be compared systemically and in quarter milk samples. This project is ongoing at a 700-cow dairy in Berlin, Pennsylvania. Samples are being collected on 125 post-partum cows 20-26 DIM, at breeding (55-60 DIM), and pregnancy check (90-95 DIM). At each time point, blood samples and quarter milk samples are taken with uterine cytology and culture samples taken 20-26 DIM. This is a joint project with Advanced Animal Diagnostics as quarter milk samples are being tested on the QScout Farm Lab for somatic cell and leukocyte differentials.

References

Akers, K. A. 2014. Changes in body condition from prepartum to postpartum on health and reproduction in dairy cattle. M. S. Thesis; West Virginia University.

Alsemgeest, S.P.M., H.C. Kalsbeek, T. Wensing, T.P. Koeman, A.M. Van Ederen, E. Gruys. 1994. Concentrations of serum amyloid A (SAA) and haptoglobin (Hp) as parameters of inflammatory diseases in cattle. Vet. Quarter. 16:21-23.

Barker, A. R., F. N. Schrick, M. J. Lewis, H. H. Dowlen, S. P. Oliver. 1998. Influence of clinical mastitis during early lactation on reproductive performance of Jersey Cows. J. Dairy. Sci. 81:1285-1290.

Bochsler, P.N., D.O. Slauson. 2002. Inflammation and repair of tissue. In: Slauson, D.O., B.J Cooper eds. Mechanisms of Disease: A Textbook of Comparative General Pathology, Third edition. St. Louis: Mosby. 140-245.
Gruys, E., M.J.M. Toussaint, T.A. Niewold, S.J. Koopmans. 2005. Acute phase reaction and acute phase proteins. J. of Zhejiang University Science. 6B:1045-1056.
Sheldon, I. M., D.E. Noakes, A. Rycroft, H. Dobson. 2001. Acute phase protein responses to uterine bacterial contamination in cattle after calving. Vet. Rec. 148:172-175.
Skinner, J. G., R.A.L. Brown, L. Roberts. 1991. Bovine haptoglobin response in clinically defined field conditions. Vet. Rec. 128:147-149.

6. Usefulness of Findings:

Findings with micro RNAs and zinc finger proteins expand our knowledge of regulation of oocyte maturation and early embryogenesis. Greater understanding of gene expression during folliculogenesis and early embryogenesis will enable solutions to early embryonic loss that continues to limit reproductive success.

Increased emphasis on immunological phenomena and changes in cytokines in relation to mastitis as an agent of embryonic loss in dairy cattle will characterize the work by Dr. Dailey and his students.

7. *Plan for Next Reporting Period*:

Objective 1

Studies to confirm the DNA-binding properties of ZNFO, and its interacting proteins especially KAP1 will be continued in the next year. (J. Yao, Leader).

Further studies of interference with early pregnancy in the sheep model of bovine mastitis and analyses of changes in white blood cell types in postpartum, lactating dairy cows in contrast to heifers will be carried out during the coming year (R. Dailey, Leader).

8. Opportunities for training and professional development

Eight graduate students have received a variety of educational experiences ranging from animal husbandry to sophisticated techniques in biochemical genetics and cell culture in the project. They have had numerous opportunities to present their findings, both orally and in writing.

9. How results have been disseminated to target audience

Publications have been listed as products above, including presentations at scientific meetings. Data have been presented in student thesis and dissertation defenses, producer meetings and university classes and seminars.

Cornell University Experimental Station 2014 Annual Report (NE1227): Ovarian Influences on Reproductive Success in Ruminants

PI-Julio Giordano, Department of Animal Science

Participants (FTE)
Individuals working on the project: PI – Julio Giordano (0.5), Graduate students at Cornell – Robert Wijma (1.0) and Matias Stangaferro (0.5), technician – Susanne Pelton (1.0), visiting scholar – Mohammed ElMetwally (0.5), undergraduate students (2.0).

Target Audience: dairy producers, bovine practitioners, and dairy industry consultants working in reproductive management. Scientists and graduate students working in reproductive physiology and management.

Efforts: reproductive physiology research with lactating dairy cattle, applied reproductive management research in commercial dairy farms, peer-review publications, outreach activities with multiple dairy industry stakeholders, classroom instruction

Products:

Peer-reviewed abstracts published in 2014 reporting research from this project

Wijma R., M.L. Stangaferro, J.R. Branen, R.G. Sasser and J.O Giordano. 2014. Altered Ovarian Dynamics in Lactating Dairy Cows Undergoing Embryonic Mortality. Abstract in Press. J. Dairy Sci. Volume 97, E-Supplement 1.

Giordano J.O., R.D. Watters, R. Wijma, and M. L. Stangaferro. 2014. Reproductive Performance of Lactating Dairy Cows after Resynchronization with Ovsynch or a Program Aimed to Maximize Artificial Insemination in Estrus and Fertility of Timed Artificial Inseminations based on Ovarian Structures. J. Dairy Sci. Volume 97, E-Supplement 1.

Proceedings published in 2014 reporting research from this project

Giordano J.O. Estrus Detection Programs and Economics of Monitoring Systems. Dairy Cattle Reproduction Council Annual Meeting. Salt Lake city, Utah. November 12-13th, 2014.

Other products

Projects

Our research has been instrumental to accomplish our goal of helping dairy farms provide U.S consumers a more affordable and abundant supply of dairy products through improved dairy herd reproductive performance, farm sustainability, and profitability. Optimal timing and establishment of pregnancy in dairy cattle is critical because it assures the long term success of dairy farms. Our research is aimed at providing a better understanding of the biological processes

that control ovarian function and pregnancy. Our goal is to use our knowledge of these biological processes to develop innovative reproductive management strategies that can be implemented by commercial dairy farms. Thus far, our fundamental research has allowed us ***to better understand the implications of early pregnancy losses on ovarian function in lactating dairy cows and the consequences of altered ovarian dynamics on subsequent response of cows to reproductive management protocols***. Also, our research has been instrumental to improve management practices that enhance reproductive efficiency of dairy cows.

1) Project lead by Robert Wijma

Altered Ovarian Dynamics in Lactating Dairy Cows Undergoing Embryonic Mortality. For this experiment conducted at the Cornell University Dairy farm our objective was to characterize ovarian dynamics in lactating dairy cows undergoing embryonic mortality. Cows (n=62) received timed AI at 60 to 79 days in milk after synchronization of ovulation. At AI, cows were randomly assigned to AI with regular semen (n=52) or extender only (n=10; Cycling; **CY**) to create a group with no chances of pregnancy as a negative control. Blood was collected at specific intervals from 14 to 42 d after AI to determine concentrations of hormones indicative of ovarian function (progesterone & estradiol) and markers of pregnancy (interferon stimulated genes and pregnancy specific protein B-PSPB). Transrectal ultrasound was performed daily to assess ovarian function. Cows were considered: 1) pregnant (**PG**; n=18) if a viable embryo with a heartbeat was observed, 2) embryonic mortality (**EM**; n=6) when a viable embryo or its heartbeat was no longer observed, and/or PSPB concentrations were initially above and then below 0.8 ng/mL, and 3) Non-pregnant (**NP**; n=28) when no viable embryo was observed and PSPB concentrations remained below 0.8 ng/mL. Preliminary results based on classification of pregnancy status with ultrasound and PSPB (data for interferon stimulated genes still not incorporated) have shown that the percentage of cows with complete luteal regression (**CLR**; important parameter to determine ovarian function) was affected (P=0.02) by group (Cycling and NonPregnant=100 vs 66.7% for Embryo Mortality) and occurred later (P<0.01) in EM 39.5±2.1) than in CY and NP cows (20.2±1.3 and 22.9±0.8). At 18 d after AI, NP cows had greater P<0.05) progesterone (**P4**) than CY cows whereas EM and PG cows had greater (P<0.05) P4 concentrations than CY and NP cows indicating delayed luteal regression. Among cows with CLR, the percentage ovulating was similar (P=0.21) for CY, NP, and EM (90.0, 85.7, and 0.0%) cows. The interovulatory interval (**IOI**) was affected by group (P<0.01). Cycling cows had the fewest days to ovulation (22.9±0.96) followed by NP cows (25.8±0.6) whereas EM cows had the longest interval to ovulation (40.0±2.0 d). Days from complete luteal rgeression to ovulation was similar (P=0.52) for CY (5.0±0.9), NP (6.0±0.6), and EM (7.0±1.9) cows. Ovulatory follicle growth rate for the 5 d preceding ovulation was similar (P=0.28) for all groups (CY=1.6±0.2, NP=1.5±0.1 and EM=0.8±0.5 mm/day). Likewise, diameter at ovulation was similar (P=0.77) for all groups (CY=22.7±1.1, NP=23.9±1.8 and EM=24.8±3.8 mm). Thus, cows with embryo mortality were less likely to undergo complete luteal regression, and had extended IOI. These data suggest that the observed differences in IOI were due to delayed luteal regression rather than alterations in follicular wave dynamics. The longer IOI for NP than CY cows may have been caused by undetected earlier embryo mortality with ultrasound and PSPB. In summary, we conclude that early embryonic mortality lead to altered ovarian function in lactating dairy cows which may explain the poor reproductive performance of previously inseminated cows in commercial dairy operations.

2) Project lead by Julio Giordano

Reproductive performance of dairy cows managed with a program aimed at increasing insemination of cows in estrus based on increased physical activity and fertility of timed artificial inseminations. For this large field study conducted at a 1000-cow commercial farm in NY we evaluated a program to manage nonpregnant cows after a previous AI aimed at increasing inseminations after a detected estrus and improve the fertility of cows not detected in estrus that must receive timed AI (TAI). This program was compared to a typical program that combines AI in estrus and TAI with the Ovsynch protocol after non-pregnancy diagnosis. Cows in the control (**CON**) group were managed with a typical program that combined AI after increased physical activity (**AIAct**) as determined by automated activity monitors and TAI after the Ovsynch protocol. After non-pregnancy diagnosis (**NPD**) by transrectal ultrasonography (**TUS**) at 31 ± 3 d after AI cows received the following treatments: 1) CON (n = 634): AIAct any time after a previous AI and resynchronization with the Ovsynch-56 protocol (GnRH-7 d-PGF2α-56 h-GnRH-16 h-TAI) 1 d after NPD or 2) TRT (n = 616): cows with a corpus luteum (**CL**) ≥ 20 mm (**TRT-CL**) received a PGF2α (**PGF**) injection 1 d after NPD whereas cows with no CL or a CL < 20 mm (**TRT-NoCL**) received a GnRH injection 3 d after NPD. Cows in TRT-CL and TRT-NoCL not AIAct were enrolled in a 5 d-Ovsynch + Progesterone (**P4**) protocol (GnRH + CIDR-5 d-PGF + CIDR removal-24 h-PGF-32 h-GnRH-16 h-TAI) 9 and 7 d after the PGF or GnRH injection, respectively to receive TAI. The hazard of pregnancy up to 270 DIM was similar for cows in the CON and TRT group (HR 1.07 95% CI 0.95 to 1.21). Median days to pregnancy for the CON and TRT group were 111 and 110 d, respectively. Thus, in spite of increasing the proportion of cows AIAct (29 and 10% for TRT and CON), median days to insemination after NPD were greater for cows in the TRT (17 d) than the CON (10 d) group which coupled with similar fertility to AIAct and TAI failed to improve overall reproductive performance. A low proportion of cows with a CL at NPD (65.2%) and a poor response to PGF may explain the poor estrus detection efficiency in the TRT group. We concluded that although more cows received AI in estrus with the strategy that promotes estrus expression, time-to-pregnancy during lactation was similar for both programs. The results of this study support the notion that dairy farms have the option to either select a strategy that attempts to increase the proportion of inseminations based upon detection of estrus after NPD or else use a more aggressive resynchronization of ovulation strategy that assures re-insemination of cows within 10 d of NPD. Thus, a practical implication of this research to the dairy industry is that dairy farms have the choice to select a strategy that increases the proportion of cows inseminated in estrus (important to numerous farms) or a program that favors TAI. Our research shows that when the former is used, it is imperative that a TAI protocol is included immediately after completion of the period intended to inseminate cows in estrus. This is even more relevant for farms that due to biological limitations from the lactating dairy cow or the myriad of environmental and management factors that affect estrus expression and detection and therefore cannot detect a high proportion of cows in estrus after NPD.

3) Project lead by Mohammed Eltmewally (Visiting Scholar form Egypt)

Prediction of Corpus Luteum Functionality with B-mode and Power Doppler Ultrasonography during the Ovsynch Protocol in Lactating Dairy Cows.

Our objective was to explore criteria to define corpus luteum (CL) functionality using B-mode ultrasound (US) and luteal blood flow (BF) in lactating Holstein cows during the Ovsynch protocol. Cows (n = 42) at Cornell University received the Ovsynch protocol (GnRH-7 d-PGF2α-56 h-GnRH). Examination of the ovaries with B-mode US was used to determine area of the CL (ACL) and power Doppler US (Sonosite EDGE) was used to determine CL blood flow. Blood was collected to estimate circulating progesterone (P4) concentrations. Cows were examined at the time of the PGF2α and second GnRH (G2) injection of Ovsynch. Luteal blood flow was assessed by measuring area of the CL with colored pixels from power Doppler US images using Image J. For cows with more than one CL all data was added to include all CL tissue. Parameters calculated included total CL tissue area (mm^2) with BF (ACLBF), percent of CL with BF (PCLBF; total proportion of colored area of the CL), and total number of colored pixels (CLPIX) per mm^2. For PCLBF calculation, the area of CL cavity was subtracted from the total CL area. A functional CL was defined as P4 ≥ 1 ng/mL. Only cows with a functional CL at PGF2α (n = 41) and a non-functional CL at G2 (n = 32) were used for statistical analyses conducted using MedCalc. ANOVA was used to evaluate CL parameters at PGF and G2 whereas Receiver Operating Characteristic (ROC) curves were used to determine the ability of CL parameters to predict CL functionality. ANOVA indicated that at the time of PGF2α, P4 (7.4 vs. 0.3 ng/mL; P<0.001), ACLBF (13.5 vs. 3.2 mm^2; P<0.001), PCLBF (20.4 vs. 7.5%; P<0.001), CLPIX number (12,893 vs. 8,590; P<0.001), and ACL (47.4 vs. 42.1 mm^2; P<0.001) was greater than at G2. ROC analysis indicated that: 1) >5.3 mm^2 of ACLBF predicted CL functionality with a sensitivity (Se) of 92.7% and specificity (Sp) of 90.3% (AUC = 94.5% CI 87-99%). 2) >12% PCLBF predicted CL functionality with a Se of 80.5% and Sp of 90.0% (AUC = 88.7% CI 80-95%). 3) >11,961 CLPIX predicted CL functionality with a Se of 61.0% and Sp of 90.3% (AUC = 76.7% CI 65-86%). 4) >60.8 mm^2 for ACL predicted CL functionality with a Se of 68.3% and Sp of 90.3% (AUC = 83% CI 72-91%). These data suggest that among multiple CL parameters evaluated with B-mode and power Doppler US, total area of the CL with BF and proportion of CL area with BF may present the highest predictive ability to determine the functionality of a CL during the Ovsynch protocol in lactating dairy cows.

Opportunities for training and professional development

Graduate student and laboratory technician training - This research has been fundamental for training graduate students and technicians in our laboratory on molecular biology, laboratory assays, use of statistical software, use of dairy management software, and reproductive techniques. Because a major component of our projects required determination of gene expression in blood cells, graduate students and technicians were trained on RNA extraction and RT-qPCR. As a result of this training our laboratory will establish RT-qPCR protocols for our current and future research. Ongoing projects under this MRP will continue to employ molecular biology techniques to be conducted in our lab. Students have also received formal training and performed the following: radioimmunoassay, transrectal reproductive ultrasonography with B-mode and Doppler ultrasound, synchronization of ovulation for timed artificial insemination.

Undergraduate student training - students (n = 5) have been involved with on-farm data collection, data processing, sample processing (blood and tissues), and basic aspects of laboratory techniques. For example some students were trained on blood sampling, transrectal

ultrasound, and use of dairy management software. These students have provided support to graduate students, technicians, and the PI. When possible have participated of experiment planning and evaluation in weekly laboratory meetings. The PI has directly worked with students to provide training.

Research collaborations - with the reproductive biology lab form Dr. Troy Ott at Penn State University. Through our well-established collaboration graduate students from their lab have received additional training on molecular biology techniques and reproductive physiology (in monthly meetings). Our collaborators have also trained members of our laboratory on molecular biology techniques. For the last 6 months we had discussion meetings approximately every 2 mo and we are working together on publishing our work. We have also established collaboration with a private company which conducted pregnancy proteins analysis for our research studies.

On-farm research collaboration & training- we have conducted research at a commercial farm (~1,000 milking cows) in the state. For these research farm personnel was trained on various reproductive techniques (ultrasound, blood sampling, synchronization protocols). We have forged a strong relationship with the farm and continue to provide management advice.

Dissemination to Target Audiences

During the reporting period the target audience for the outcomes of our MRP funded research has been broad.

-Dairy producers: the results of our research have been presented in formal and informal settings to dairy producers across the state. The PI and graduate students have presented at conferences and meetings with producers in attendance. Also, the PI has worked directly with dairy farms to help them establish the reproductive management program for their farms using the results of our research as a platform for program design. Our research data has been very useful to help producers understand some of the implications of pregnancy losses on the performance of their herds. Producers have been one of the most receptive audiences as they can use the information generated to explain some of the events that they observe on a daily basis on their farms. The results of our research projects aimed at defining new reproductive management strategies based on the ovarian status of previously inseminated cows has been instrumental in helping producers make adjustments to their own farm reproductive programs. We have worked with farms of all size ranging from 25 to 2,000 cows.

-Industry consultants and Extension [includes bovine veterinarians, consultants from allied industries (e.g., AI industry, pharmaceutical), and extension educators from Cornell Cooperative Extension and PRO-Dairy]. The PI has presented the preliminary results of our research in multiple conferences, workshops, and extension meetings. This is a very important audience because they are multipliers of the information generated as they work on a daily basis with numerous producers representing all farms sizes and types.

-Scientific community: We have presented the results of our research through abstracts, posters, and oral presentations at the Annual Meeting of the American Dairy and Animal Science Association. This is the largest gathering of scientist in the field with approximately 3,000 scientists in attendance. Our research has also been presented at the Dairy Cattle Reproduction Council 2014 Annual Meeting. The PI had a poster presentation at this meeting with producers, veterinarians, AI industry, and reproduction industry consultants. Preliminary results of our

research have also been presented to members of the Multistate Regional Project (NE-1227) during the group's annual meeting at Penn
State University.

Selected Presentations:

Giordano J.O. Reproductive Management: Practical Strategies and Research Update. Northern New York Dairy Institute. Watertown, Lowville, and Chazy, New York. December 16th, 17th, and 18th, 2014.

Giordano J.O. Ohio Dairy Health and Management Certificate Program: Dairy Cattle Reproduction with Emphasis on Transition Cow Management in Confinement Systems. 1. Activity Monitors: Managing 1st and 2nd and Subsequent AI Services. College of Veterinary Medicine, The Ohio State University, Columbus, Ohio. August 7th, 2014.

Giordano, J.O. Reproductive Physiology Update. Summer Dairy Institute. College of Veterinary Medicine, Cornell University. Ithaca, New York. June 24th, 2014.

Giordano, J. O. Dairy Health Management Certificate Program. 1. Reproductive Management I: Practical Strategies and Research Update, 2. Pregnancy Losses and Non-Pregnancy Diagnosis, 3. Reproductive Management II: Integrating Activity Monitoring into Reproductive Management Programs. Ontario College of Veterinary Medicine, Guelph Ontario. June 3rd to June 5th, 2014.

Giordano J.O. Dairy Day at Miner Institute. Evaluating Reproductive Management Strategies and their Profitability. Chazy, NY. December 3rd, 2013.

Plan for next reporting period

Our work to accomplish the goals of the project is underway. We are currently conducting further laboratory tests to have more data available for publishing, planning other studies, and grant proposal writing. We continue to analyze our data and writing manuscripts for publication in peer reviewed journals. We are at the moment defining the final details of our next set of experiments to be performed at the Cornell University Dairy farm and commercial dairy operations. We are building upon our findings from our previous research to design new studies (at least 3 large studies) to further our understanding of ovarian function in cows that lose their pregnancy and design new management protocols for dairy farms. We are also testing some new techniques and providing proofs of concept for procedures that have not been used before for the type of research we are conducting. The fundamental research data generated is also being used to test innovative management protocols that may continue to improve the reproductive performance of dairy cattle. We also plan on continuing our efforts to seek additional funding from USDA as well as other state and private funding sources.

Multistate project NE-1227

Annual report 2015 – Cornell University

W.R. Butler

Participants (FTE)
Individuals working on the project: PD – W.R. Butler (0.2); Technical support – Susanne Pelton (0.4)

Target Audience
Target Audiences: One target audience reached during this reporting period was the group of scientists and their graduate students from other research stations participating and collaborating in the Multistate Project NE-1227. This group met in June 2014 for the annual meeting held at Pennsylvania State University for exchange and discussion of new research data and methodology and planning for future specific projects. The current meeting, June 2015, is being held at University of New Hampshire.

The other audiences reached were scientists and graduate students interested in reproductive physiology of cattle and attending the 2014 annual meeting of the American Dairy Science Association. This large conference provides venues for presentation of abstracts (posters and platform presentations) describing research results conducted on this project at Cornell University.

Efforts: peer-reviewed manuscripts; conference and meeting presentations.

Products

Vieira-Neto, A., R. O. Gilbert, W. R. Butler, J. E. P. Santos, E. S. Ribeiro, M. M. Vercouteren, R. G. Bruno, J. H. J. Bittar, and K. N. Galvao. Individual and combined effects of anovulation and cytological endometritis on the reproductive performance of dairy cows. *J Dairy Sci.* 97:5415-5425, 2014.

Other Publications, conference papers and presentations

N/A

Accomplishments
What was accomplished
Objective A: Identify intracellular signaling pathways and gene expression regulatory mechanisms within the ovary, embryo, or female reproductive tract that promote oocyte growth and maturation, fertilization, early embryonic development, and establishment and maintenance of pregnancy

Blood samples and phenotypic data on reproductive performance were collected from lactating dairy cows at 3 stations (NY, PA, and NH) following the protocol designed at the 2013 annual meeting. **DNA and SNP Analysis for Fertility in Dairy Cattle: NE-1227 collaborative project.** DNA was extracted and cows were genotyped for a *AluI* polymorphism in the growth hormone receptor (GHR) gene following the procedures published previously by Schneider et al.(Association between growth hormone receptor AluI polymorphism and fertility of Holstein cows. Theriogenology 80:1061-1066, 2013).

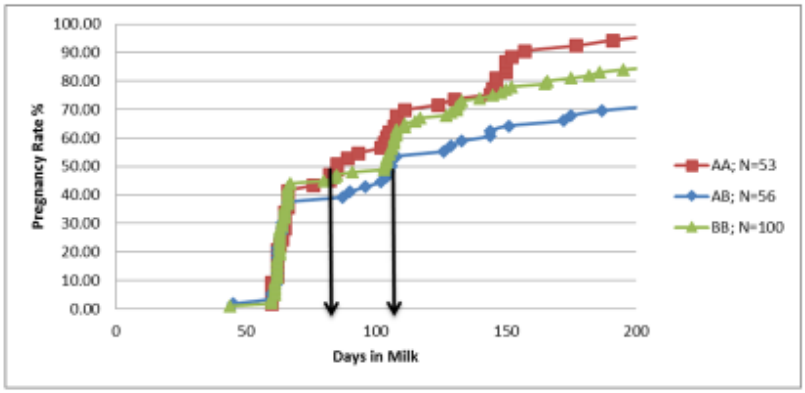
Comparison of GHR AluI Genotypes among 3 research herds

	% (N)		
Genotype	Cornell U.	Penn St. U	U. New Hampshire
A (-) allele	0.39	0.51	0.53
B (+) allele	0.61	0.49	0.47
AA	25% (53)	42% (41)	48% (17)
AB	27% (56)	18% (18)	9% (3)
BB	48% (100)	40% (40)	43% (15)
Cows	209	99	35

Comparison of Fertility among herds

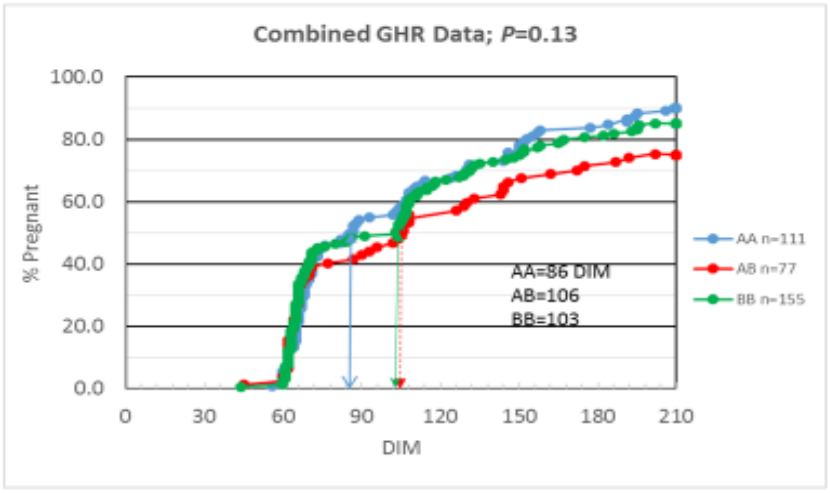
Station	Preg% to 1st AI	Median DIM @ pregnancy	%Non-Preg @ 210 DIM
CU	84/209 (40%)	103	33/209 (16%)
PSU	53/99 (53%)	73	13/99 (13%)
UNH	13/35 (37%)	109	7/35 20%)

Cornell cows GHR Fertility data



Survival curves of pregnancy rate by GHR AluI genotype. The vertical arrows represent the median number of days for achievement of pregnancy in each of the groups. The value for AA cows was 85 DIM, while AB and BB cows is essentially equivalent (106 days and 103 days, respectively; P=0.025).

Combined data on GHR and Fertility in cows



Survival curves of pregnancy rate by GHR AluI genotype for combined data from 3 stations. The median number of days to pregnancy for each genotype are shown by arrows and text box. The data was not statistically significant, but may become a trend with additional data.

Opportunities for training and professional development
Undergraduate students were trained by the PI and a technician to collect and extract DNA from blood samples and to carry out PCR for genotyping of single nucleotide polymorphisms (SNPs) in dairy cows.

How have the results been disseminated to communities of interest?
The results have been published in peer-reviewed scientific journal articles and abstracts and, thus, made available to other scientists, veterinarians, and agriservice consultants with interests in improving reproductive performance of dairy cows.

What do you plan to do during the next reporting period to accomplish the goals?

Objective A

1) Complete the GHR genotyping of 100 dairy cows for comparison and analysis of phenotypic data for DMI and energy balance during early lactation.

2) At the annual meeting of the Multistate project committee in June 2013, a collaborative project was discussed and initiated at multiple stations (n=5). The objective: to obtain blood samples for DNA genotyping and pregnancy/fertility phenotype information from ~1000 lactating dairy cows. Thus far, 375 samples were collected and DNA extracted; IGF-I SNP analyses will be completed on these samples.
    - The research protocol was developed for this project and was distributed to collaborating members,

Major Changes
N/A

Impact statements:

1) The presence of the growth hormone receptor (GHR) AluI(-) allele in Holstein cows is associated with increased serum IGF-I concentrations and a shorter calving to conception interval during lactation ***ie*****. higher fertility.**

ANNUAL REPORT OF COOPERATIVE REGIONAL RESEARCH PROJECT NE-1227

Project: Ovarian influences on reproductive success in ruminants

Participants (FTE): At Cornell University, Department of Animal Science: PI- Susan Quirk (0.1), Research Support Specialist- Robert Cowan (0.1)

Target audience:
Research scientists, producers
Efforts: Laboratory research, training of undergraduate and graduate students

Products:
Publications – none, work is in data collection and analysis phase.

Data collection and analyses: Oocytes were collected from large batches of ovaries obtained at an abattoir. Oocytes were cultured to allow collection of both germinal vesicle stage and mature oocytes. An assay for sonic hedgehog messenger RNA was designed. Oocyte RNA was prepared and used in real-time RT-PCR assays.

Other Products:

Accomplishments:

NE1227 Objective A: Identify intracellular signaling pathways and gene expression regulatory mechanisms within the ovary, embryo, or female reproductive tract that promote oocyte growth and maturation, fertilization, early embryonic development, and establishment and maintenance of pregnancy

The overall goal is to contribute to the production of high quality bovine oocytes. The hypothesis tested is that sonic hedgehog is a maternal effect gene with a role in either meiotic maturation or early embryonic development. We found that sonic hedgehog messenger RNA was not expressed at detectable levels in the bovine oocyte at either germinal vesicle stage or at metaphase II. These findings do not support the hypothesis. We plan to redirect focus to other aspects of hedgehog signaling in somatic cells of bovine ovarian follicles that are hypothesized to influence follicle development.

Opportunities for training and professional development

A technician and graduate student in the lab gained expertise in isolation, culture and gene expression analysis of bovine oocytes

How results have been disseminated to target audience

Presentation of results at annual NE1227 meeting

Plan for next reporting period

Explore systems for culture of bovine ovarian tissues and cells to best address the goal of determining the role of hedgehog signaling in somatic cells of the follicle and surrounding cells in supporting follicle development. Possibilities are whole follicle, hemisected follicles, dispersed thecal cells and granulosa cells, whole theca tissue, and pieces of ovarian cortex from fetal calves.

Determine the significance of changes in expression of HH pathway components with follicle development. For example: 1. determine the signal(s) initiating hedgehog signaling at the primary follicle stage; 2. determine the role of declining hedgehog signaling in preovulatory follicles in response to the preovulatory LH surge.

Determine downstream pathways stimulated by hedgehog signaling in somatic cells of the follicle.

Major Changes

The focus of our work will relate primarily to objective B of NE1227 **[Identify inter-cellular interactions between somatic cells of the ovary, somatic cells and germ cells, or somatic cells and the embryo that promote follicular growth, oocyte maturation, early embryonic development, and establishment and maintenance of pregnancy]** whereas our previous focus was on objective A.

Support

NE1227 multistate funding

Annual Report of Cooperative Regional Research Project NE-1227
Cornell University Agricultural Experiment Station
2015

PART II OF THE CORNELL UNIVERSITY REPORT
J.E. Fortune, M.Y. Yang, and J.J. Allen

Title: Ovarian Influences on Reproductive Success in Ruminants

Objective A: Identify genetic, morphological and physiological attributes of the ovary considered to improve fertility in ruminants

A. Introduction

In the current 5-year project, our efforts are focused on steroid-dependent mechanisms that contribute to the temporal regulation of follicle formation and activation. Previously our lab developed an experimental model for studying early follicular development in cattle and baboons, using small pieces of ovarian cortex in organ culture (Wandji et al., 1996, 1997). More recently, we have shown that in vitro estradiol (and progesterone) inhibit both follicle formation and acquisition of follicular competence to activate (Yang & Fortune, 2008). Because estradiol (E2) and progesterone (P4) production by bovine fetal ovaries declines dramatically in the 10 days preceding the start of follicle formation (day 80-90) and more slowly during the period when follicles acquire competence to activate (day 90-140), we hypothesize that, in vivo, fetal ovarian steroids play a role in regulating follicle formation and competence to activate. Understanding some of the mechanisms of estradiol's actions on the fetal ovary is the major goal of our part of the current Multistate project. The experiments described below were designed to test the hypothesis that the effects of estradiol are mediated via the nuclear estrogen receptors (ESR1 and/or ESR2). In other experiments, effects of activin on steroidogenesis and follicle formation and activation were determined.

B. Experiment 1. Effects of estrogen receptor agonists and antagonists on follicle formation and activation in vitro

We have shown previously that E2 inhibits both follicle formation and capacity to activate in ovarian cortical pieces from 90 to 140-day-old fetuses and that inhibition of activation is associated with inhibition of the progression of meiotic prophase I by E2. However, we know little about the mechanism(s) by which E2 exerts these effects. This experiment is our first step towards understanding those mechanisms.

Hypothesis: The inhibitory effects of E2 on follicle formation and acquisition of the capacity to activate are mediated through one or both nuclear estrogen receptors (ESR1 and ESR2).

Protein and mRNA for both ESR1 (ERα) and ESR2 (ERβ) were detected in various cell types in fetal bovine ovaries beginning around day 45 of gestation (Garverick et al., 2010; Burkhart et al., 2010). Therefore, it seems logical to hypothesize that these receptors are involved in the actions of fetal E2 on follicle formation and activation. Last year we presented results showing that the general ESR antagonist ICI 182,780 can block estradiol's inhibitory effects on follicle formation and activation. Since then we have tested both agonists and antagonists of ESR1 and ESR2 to determine which nuclear ER(s) is involved in mediating the

actions of estradiol.

Methods and Experimental Design: Ovaries were obtained from bovine fetuses (110-134 days old) at a local slaughterhouse, with age estimated by crown-rump length (Evans and Sack, 1973). The ovarian cortex was isolated and cut into small pieces which were distributed to the wells of 24-well plates (2 pieces/well, 2 wells per treatment in each experiment) and cultured for 10 days with control medium (containing insulin), E2 (1 μM), the ESR1 and ESR2 agonists PPT and DPN, respectively and the ESR1 and ESR2 antagonists PPT and PHTPP, respectively (all at 1 μM). Cortical pieces were fixed either immediately (day 0 controls) or at the end of culture, embedded in LR white plastic, and serially sectioned. Every twentieth section was analyzed by histological morphometry for numbers of primordial and primary follicles. The experiments were repeated with 3 fetuses. The results were analyzed by ANOVA, followed by Duncan's multiple range test, if ANOVA indicated significant effects of treatment.

Results: The results suggest that both ESR1 and ESR2 are involved in mediating the inhibitory effects of E2 on follicle formation, but that only ESR2 is involved in E2's effects on follicle activation (Fig. 1 and 2). However, the results for ESR2 are clearer cut than those for ESR1.

Conclusions: Based on experiments with agonists and antagonists, the effects of estradiol on follicle formation and capacity to activate appear to be mediated primarily through ERS2.

C. Experiment 2. Effects of activin on fetal ovarian steroidogenesis and on follicle formation and activation in fetal bovine ovaries

Hypothesis: Activin will increases FSH-stimulated production of E2 and may affect follicle formation and/or activation.

Bovine fetal ovaries have protein and mRNA for activin A and its receptors (data not shown), suggesting a role for activin in early folliculogenesis in cattle. In granulosa cells, activin increases FSH-stimulated aromatization

Methods and Experimental Design: Ovaries were obtained from 89-110 day-old bovine fetuses at a local slaughterhouse, with age estimated by crown-rump length (Evans and Sack, 1973). This is around the time follicle formation is initiated (around day 90) and about 50-30 days before the first primary follicles appear (around day 140). For Exp. 2a (steroidogenesis) the ovaries were cut into pieces and for Exp. 2b (follicle formation and activation) the ovarian cortex was isolated and cut into small pieces. Ovarian or cortical pieces were distributed to the wells of 24-well plates (2 pieces/well, 2 wells per treatment in each experiment). In Exp. 2a, duplicate cultures were treated with control medium or with FSH (50 ng/ml) or T+FSH (0.1 μM and 50 ng/ml, respectively), each in absence or presence of activin A (100 ng/ml). In Exp. 2b, duplicate cultures were treated with control medium (TS+, insulin (ITS+), kit ligand (100 ng/ ml), BMP4 (50 ng/ml), or activin A (100 mg/ml).

In Exp. 2a, medium was collected every other day and measured for E2 and P4 by RIA. In Exp. 2b, cortical pieces were fixed either immediately (day 0 controls) or at the end of culture, embedded in LR white plastic, and serially sectioned. Every twentieth section was analyzed by histological morphometry for numbers of primordial and primary follicles. The experiments were repeated with 3 fetuses. The results were analyzed by ANOVA, followed by Duncan's multiple range test if ANOVA indicated significant effects of treatment.

Results: In Exp. 2a, both activin and FSH stimulated progesterone production and the

combination was no more effective than either alone (Fig. 3). Testosterone inhibited the stimulatory effect of FSH on P4, as expected, but we were surprised to observe that activin overcame the inhibitory effect of testosterone. In contrast, activin had no effect on E2 production either alone or in combination with FSH or FSH + T (Fig. 3).

At the start of culture (day 0) in Exp. 2b and after 10 days in control medium (TS+), almost all follicles were primordial, but after 10 days some follicles cultured with insulin had activated to become primary follicles (Fig. 4). In addition, insulin increased the total number of follicles about 2.5-fold compared with the day 0 or day 10 controls. Neither KL nor BMP4, which stimulate activation in fetal tissue after day 140, had an effect on follicle formation or activation compared with the day 10 control. In contrast, in the presence of activin the total number of follicles and the number of primary follicles was not different from the insulin control.

Conclusions: The results of Experiment 2a show that activin A had dramatic and selective effects on steroidogenesis by fetal bovine ovarian pieces. It stimulated P4 and overcame the inhibitory effects of testosterone on FSH-stimulated P4, but had no effect on E2 production. In Exp. 2b, follicles only formed in the presence of insulin or activin and both factors also promoted activation. In contrast, KL and BMP4 which stimulate activation in older fetal ovaries (after day 140) did not affect follicle numbers or activation. Together these experiments show that activin may play a significant role in regulating both steroidogenesis and early follicle development in bovine fetal ovaries. Since activin stimulates progesterone production and progesterone can inhibit follicle formation and activation, the effects of activin on steroidogenesis vs. follicular development seem contradictory, based on what we know now.

D. Summary of findings

The results presented provide evidence that the effects of estradiol on bovine follicle formation and capacity to activate are mediated through binding to a nuclear receptor, with evidence particularly strong for a role for ESR2. Activin A had effects on both early follicular development (stimulation of follicle formation and activation) and also on steroidogenesis by fetal bovine ovarian tissue, with effects on progesterone, but not estradiol, production.

E. Usefulness of Findings. Primordial follicles comprise the "follicular reserve" which will supply the female with follicles and gametes throughout her reproductive life. Thus a greater understanding of how the pool of resting follicles is formed and of the signals that allow or inhibit activation and subsequent growth is of potential practical importance.

F. Supplemental Funding

USDA (NRICGP, #2008-35203-05989); 9/30/09-8/31/014; PI - 15% effort

"Effects of Fetal Steroids on the Formation of Ovarian Follicles and on their Competence to Initiate Growth"

F. References

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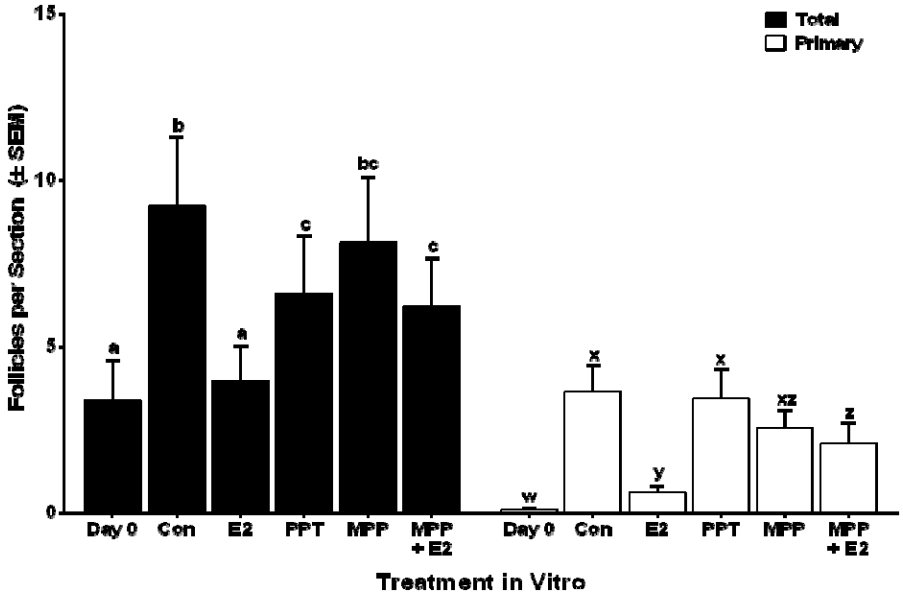

Fig. 1. Effects of estradiol (E2), the estrogen receptor 1 (ESR1) agonist (PPT) and ESR1 antagonist (MPP) on follicle formation and activation in cortical pieces from fetal bovine ovaries. Pieces were analyzed by histological morphometry on Day 0 or after 10 days of culture in control medium (Con) or with 1 μM each of E2, PPT, MPP or MPP+E2. Data are total (primordial + primary) and primary follicles per section (± SEM). Means with no letters in common (total: a-c; primary: w-z) are significantly different. (N = 6 cultures, 2 from each of 3 fetuses between 110 and 134 days old)

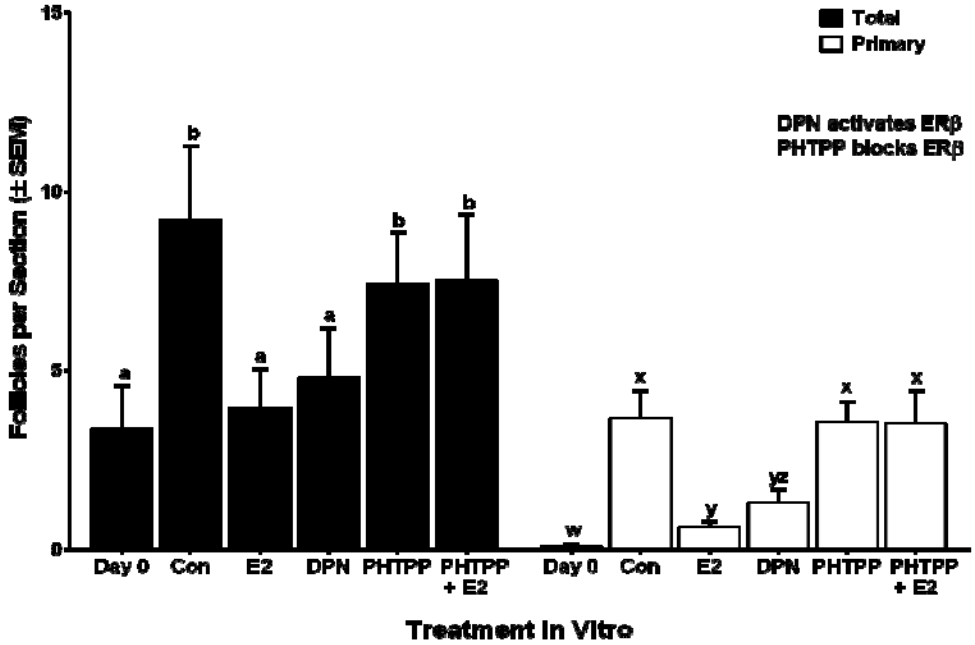

Fig. 2. Effects of estradiol (E2), the estrogen receptor 2 (ESR2) agonist (DPN) and ESR2 antagonist (PHTPP) on follicle formation and activation in cortical pieces from fetal bovine ovaries. Pieces were analyzed by histological morphometry on Day 0 or after 10 days of culture in control medium (Con) or with 1 μM each of E2, DPN, PHTPP or PHTPP+E2. Data are total (primordial + primary) and primary follicles per section (± SEM). Means with no letters in common (total: a, b; primary: w-z) are significantly different. (N = 6 cultures, 2 from each of 3 fetuses between 110 and 134 days old)

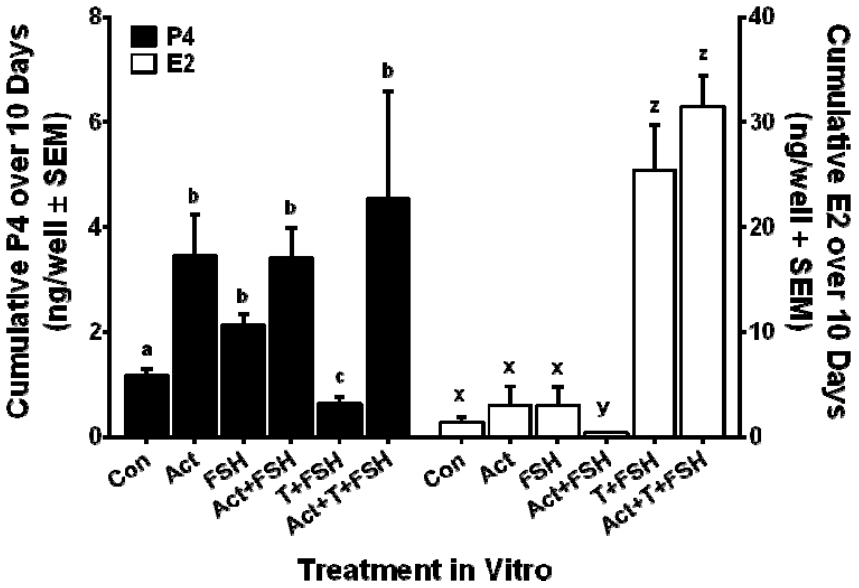

Fig. 3. Accumulation of progesterone (P4) and estradiol (E2) in cultures of pieces from fetal bovine ovaries over 10 days. Pieces were cultured in control medium (Con), with FSH (50 ng/ml) or T+FSH (0.1 µM and 50 ng/ml, respectively), each in absence or presence of activin (Act; 100 ng/ml). Data are non-transformed means ± SEM. Means with no letters in common (P4: a-c; E2: x-z) are significantly different. (N = 6 cultures, 2 from each of 3 fetuses between 89 and 109 days old).

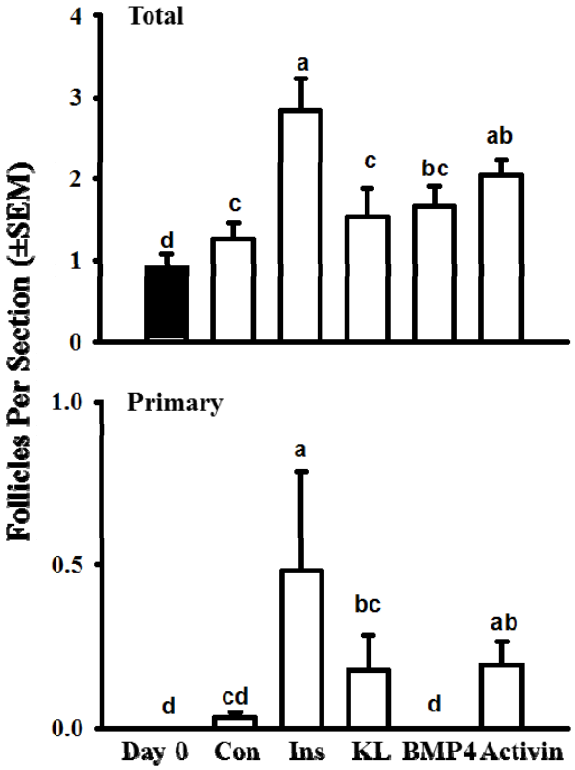

Fig. 4. Effects of insulin (Ins; 6.25 µg/ml), KL (100 ng/ml), BMP4 (50 ng/ml) and activin (100 ng/ml) on follicle formation and activation in cortical pieces from 90- to 110-day-old bovine fetuses in vitro (n = 6 cultures; 2 from each of 3 fetuses). Cortical pieces were cultured for 10 days. Means with no common letters are different ($P < 0.05$)

ANNUAL REPORT OF COOPERATIVE REGIONAL RESEARCH PROJECT NE-1227
Iowa State University
Department of Animal Science
2015 Annual Report

Project: NE-1227, Ovarian Influences on Reproductive Success in Ruminants

Participants (FTE)
Iowa State University, Department of Animal Science. Individuals working on the project: PI – Aileen F. Keating, Co-PI – Jason W. Ross. Graduate students Ahmad A. Al-Shaibi, Shanthi Ganesan, Porsha Thomas, Ben Hale, Jake Seibert.

Target Audience

Target Audiences: Scientific, agricultural and healthcare communities, Producers and commodity groups in the state of Iowa.
Efforts: conference and meeting presentations, peer-reviewed manuscripts, formal undergraduate and graduate classroom reproductive physiology instruction, experiential research learning opportunities at ISU.

Products
Journal Articles, Reviews and Book Chapters:
Nteeba, J., Sanz-Fernandez, M.V., Rhoads, R.P., Baumgard, L.H., Ross, J.W., Keating, A.F. Heat Stress Alters Ovarian Insulin Mediated Phosphatidylinositol-3 Kinase and Steroidogenic Signaling in Gilt Ovaries. Biology of Reproduction, *In Press*.

Colpoys, J.D., Abell, C.E., Gabler, N.K., Keating, A.F., Millman, S.T., Siegford, J.M., Young, J.M., Johnson, A.K. 2015. Feed efficiency effects on barrow and gilt behavioural reactivity to novel stimuli tests. Journal of Animal Science.93:1–9.

Ganesan, S., Nteeba, J., Keating, A.F. 2015. Impact of obesity on 7,12-dimethylbenz[a]anthracene-induced altered ovarian connexin gap junction proteins in female mice. Toxicology and Applied Pharmacology. 282(1):1-8. Cover Picture.

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Cruzen, S.M., Boddicker, R.L., Graves, K., Johnson, T.P., Arkfeld, E.K., Baumgard, L.H., Ross, J.W., Safranski, T.J., Lucy, M.C., Lonergan, S.M. 2015 Carcass composition of market weight pigs subjected to heat stress in utero and during finishing. (Accepted: Journal of Animal Science).

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Hale, B.J., Keating A.F., Yang, C.X., Ross J.W. 2015 Small RNAs: Their possible roles in reproductive failure. In: The Male Role in Pregnancy Loss and Embryo Implantation Failure. Ed. Richard Bronson. Springer Publishing.

Other Products

Abstracts:

Powell, E., Cunnick, J., Waide, E,. Schroyen, M., Goldman, F.D., **Ross, J.W.,** Ellinwood, N.M., Snella, E., Esch, K., Dekkers, J.C.M., Tuggle, C.K. 2014 Toward humanization of the pig; boldly going where only mice have gone before. Plant and Animal Genome XXII Conference. San Diego, CA, January 11-15.

Waide, E.H., Tuggle, C.K., Ellinwood, N.M., **Ross, J.W.,** Dekkers, J.C.M. 2014 Not all SCID pigs are created equally: two natural mutations cause Severe Combined Immuno Deficiency (SCID) in pigs. Plant and Animal Genome XXII Conference. San Diego, CA, January 11-15.

Johnson, J.S., Sanz-Fernandez, M.V., Patience, J.F., Ross, J.W., Gabler, N.K., Lucy, M.C., Safranski, T.J., Rhoads, R.P., Baumgard, L.H. 2014 *In utero* heat stress alters body composition during the early finishing phase (60 to 80 kg) in pigs. American Society of Animal Science Midwest Section Meeting. Des Moines, IA. March 17-19.

Johnson, J.S., Sanz-Fernandez, M.V., Patience, J.F., **Ross, J.W.,** Gabler, N.K., Lucy, M.C., Safranski, T.J., Rhoads, R.P., Baumgard, L.H. 2014 Effects of *in utero* heat stress and core body temperature on tissue accretion during the growing phase (30 to 60 kg) in pigs. American Society of Animal Science Midwest Section Meeting. Des Moines, IA. March 17-19.

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Scott, P.A., Fernandez de Castro, J.P., DeMarco P.J., Ross, J.W., Walters, E., Prather, R.S., McCall, M.A., Kaplan, H.J. 2014 Pro-23-His transgenic miniature swine exhibit functional and morphological characteristics similar to humans with autosomal dominant retinitis pigmentosa. 2014 Swine Biomedical Research Conference. Raleigh, NC, July 6th-8th.

Izquierdo-Rico MJ, **Ross JW**, Soriano-Úbeda C, Hernández-Caravaca I, Viera L, Matas C, García-Vázquez FA. 2014 Prostaglandin PGJ2 added to artificial insemination sperm dosage reduces the expression of the inflammatory genes

Colpoys, J.D., Abell, C.E., Gabler, N.K., Keating, A.F., Millman, S.T., Siegford, J.M., Johnson, A.K. 2015. Barrow behavioral reactivity to a human or novel object when fed low versus high energy diets. American Society of Animal Science annual meeting (poster presentation).

Cheng, L., Nteeba, J., Keating, A.F., Cui, J.Y. 2015. Impact of Obesity on the Expression of Xenobiotic Metabolism Genes in the Mouse Liver. Society of Toxicology annual meeting.

Keating, A.F. 2015. Impact of phosphoramide mustard exposure on ovarian drug transporter expression. Society of Toxicology annual meeting.

Seibert, J.T., Boddicker, R.L., Koltes, J.E., Reecy, J., Nettleton, D., Lucy, M.C., Safranski, T.J., Rhoads, R.P., Gabler, N.K., Baumgard, L.H., **Ross, J.W.** 2014 Alterations in transcriptional profile as the result of prenatal HS exposure in pigs. Plant and Animal Genome XXII Conference. San Diego, CA, January 11-15.

Accomplishments – Major findings, Key Outcomes

The long-term goal of this project is to (1) identify intracellular signaling pathways and gene expression regulatory mechanisms that promote oocyte growth and maturation; and (2) identify inter-cellular

interactions between somatic cells of the ovary (i.e. theca and granulosa) as well as somatic cells and the oocyte (i.e. granulosa and oocyte) that promote follicular growth and oocyte maturation. The rationale for this work is that thirty percent of beef cows and 50% of dairy cow pregnancies are lost due to embryonic mortality resulting in a loss of 1.4-1.2 billion dollars in the US. Problems associated with embryonic mortality may be due in part to oocytes that are not competent to be fertilized. It is our hypothesis that metabolic stressors (e.g. obesity, nutrient restriction, altered metabolic hormone levels, heat stress, environmental chemical exposures) alter the microenvironment of the oocyte during growth and maturation which impairs the developmental potential of the oocyte. Toward this goal the following have been accomplished:

Identification of the impact that thermal stress has on ovarian signaling is resulting in a mechanistic map being developed, upon which strategies to ameliorate seasonal infertility can be based.

Whole genome sequencing is contributing to understanding biological reasons for the impact of gestational heat stress on the body composition of offspring.

Tissue and blood collection has been completed from synchronized post-pubertal gilts exposed to chronic lipopolysaccharide or thermal stress.

Opportunities for training and professional development

1) Undergraduate students were provided with formal laboratory research as well as experience in live animal phase heat stress and chronic LPS infusion projects at ISU.

2) One graduate students successfully defended their doctoral dissertations.

3) Training and professional development: Students were trained by the PI and technicians in molecular biology techniques including PCR and western blotting. Additionally, students (graduate and undergraduate) gave oral and poster presentations at local, national and international scientific meetings.

4) A weekly reproductive discussion group has continued to discuss new findings in reproductive biology as well as provide students a sounding board for discussion of their own research data.

5) Graduate students served on committees at Iowa State University.

How results have been disseminated to target audience

Twenty-one scientific manuscripts were published in high quality peer-reviewed journals
Graduate students and post-docs presented 11 abstracts at the Society for the Study of Reproduction, Society of Toxicology, Plant and Animal genome and the American Society of Animal Science annual meetings.

Plan for next reporting period

Objective 1

Our work on PI3K and microRNA regulation of chemical metabolism will continue. We have a large bank that we are working through to understand infertility that results from chemical exposure, heat stress and elevated circulating lipopolysaccharide.

We are currently investigating impacts of central metabolism alterations on reproductive function. We have access to ovaries from hypoleptinemic mice, hyperinsulinemic pigs, obese pigs, and lipopolysaccharide treated pigs. In addition, we are exposing postnatal day 4 ovaries in culture to leptin, insulin and LPS to determine direct effects on ovarian function, and chemical metabolism.

6. Funding Obtained:
Iowa Pork Producers Association.
Dates pending
Advancing investigations in swine reproduction efficiency at Iowa State University
$100,000
A.F. Keating, Co-investigator

Global Food Security Consortium.
10/01/14-06/30/15.
Determination of lipopolysaccharide involvement in climate-induced infertility
$25,000 total costs
A.F. Keating, Principal Investigator

Iowa Pork Producers Association.
07/01/14-06/30/15
Investigating hyperinsulinemia and increased circulating lipopolysaccharide involvement in seasonal infertility
$55,719 total costs
A.F. Keating, Principal Investigator.

Iowa State University Internal Funding Program.
Examination of Genomic Markers Associated with Heat Stress in Pigs, 2014-2015.
$26,400 total costs
A.F. Keating, Co-investigator.

Major Changes
N/A

ANNUAL REPORT OF COOPERATIVE REGIONAL RESEARCH PROJECT NE-1227

The Pennsylvania State University
Department of Animal Science
2015 Annual Report

Project: NE-1227, Ovarian Influences on Reproductive Success in Ruminants

***Participants*:** Penn State University, Department of Animal Science, Joy L. Pate (Principal Leader), Francisco Diaz, Troy Ott

Target Audience: Researchers, farmers

Products

Journal Articles, Reviews and Book Chapters **:**

Maalouf SW, Liu W-S, Albert I, Pate JL. 2014. Regulating life or death: Potential role of microRNA in rescue of the corpus luteum. Mol Cell Endocrinol 398:78-88.

Holler TL, Dean M, Taylor T, Poole DH, Thonney ML, Thomas DL, Pate JL, Whitley N, Dailey RA, Inskeep EK. 2014. Effects of service sire on prenatal mortality and prolificacy in ewes. J Anim Sci 92:3108-3115. (listed in press last year)

Walusimbi SS, Pate JL. 2014. Luteal cells from functional and regressing corpora lutea differentially alter the function of gamma-delta T cells. Biol. Reprod. 90(6):140, 1-10. (listed in press last year)

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Tian X, Anthony K and **Diaz FJ**. Zinc regulates progesterone production in mouse cumulus-oocyte complexes and corpora lutea. Submitted.

Sun T, Pepling M and **Diaz FJ**. *Lats1* deletion causes an increase in germ cell apoptosis and follicular cysts in mouse ovaries. Submitted.

Ott, TL, Kamat, MM, Vasudevan, S, Townson, DH, Pate, JL 2014. Maternal immune responses to conceptus signals during early pregnancy in ruminants. Anim. Reprod. 11(3): 237-245.

Ott, TL, Dechow, C, O'Connor, ML 2014. Advances in reproductive management: pregnancy diagnosis in ruminants. Anim. Reprod. 11(3): 207-216.

Abstracts**:**

Maalouf, S., W-S. Liu, I. Albert, J. Pate. 2014. MicroRNA of the corpus luteum during maternal recognition of pregnancy. Society for the Study of Reproduction. Abstract 552.

Steinberger L, Pate JL. 2014. Isolation and characterization of macrophages in the bovine corpus luteum. Amer J Reprod Immunol 71 (Suppl. 1):81

Branham K, Waugh S, Pate JL. 2014. In vitro differentiation of bovine macrophages. Amer J Reprod Immunol 71 (Suppl. 1):37

J. Oh, S. Walusimbi, F. Giallongo, H. Weeks, T. Frederick, A. N. Hristov, J. L. Pate, R. J. Elias, L. Tao, and E. H. Wall. 2014. Effect of dietary supplementation of Capsicum extract on immune responses, blood cell counts, blood chemistry, and oxidative stress markers in lactating dairy cows. J. Dairy Sci. 97(E-Suppl. 1):103.

Townson DH, Diaz FJ, Ocon-Grove O, Johnson AL. 2014. A practical *in vitro* approach for the investigation of bovine granulosa cells from small follicles. *Proceedings of the 47th Annual Meeting of the Society for the Study of Reproduction*, Grand Rapids, MI.

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Gernand AD, Cantorna MT, Diaz FJ, Snyder LM, Hester JM, Vasudevan S, Kamat MM and Ott TL. 2015. Prepregnancy Vitamin D Deficiency and Placental Development in Mice. *Experimental Biology Meeting*, Boston, Massachusetts. FASEB J April 2015 29:LB259

Theses/Dissertations:

Branham, KL. 2015. Role of luteal cell-derived exosomes in communication with immune cells. M.S. Thesis. The Pennsylvania State University.

Other Products

Accomplishments

Objective A: Identify intracellular signaling pathways and gene expression regulatory mechanisms within the ovary, embryo, or female reproductive tract that promote oocyte growth and maturation, fertilization, early embryonic development, and establishment and maintenance of pregnancy

Our *working hypothesis* is that regulation of signaling pathways and gene expression in somatic and germ cells by paracrine factors and steroid hormones ensures ovarian cyclicity, the production of developmentally competent oocytes, and the regulation of early embryogenesis.

A. MicroRNA in the corpus luteum during maternal recognition of pregnancy. Samar W. Maalouf, Wansheng Liu, Istvan Albert, Joy L. Pate.

In the cow, regression of the corpus luteum (CL) is initiated by uterine release of prostaglandin $F_{2\ alpha}$, and rescue of the CL during maternal recognition of pregnancy is dependent on release of interferon-tau by the developing conceptus. The exact mechanisms that are responsible for determining the fate of the CL are unknown. Few studies have focused on posttranscriptional

regulators, in particular the role of microRNA (miRNA) in luteal rescue or regression. Using next generation sequencing, we profiled miRNA expression in the bovine CL during maternal recognition of pregnancy. In this study, CL were collected from heifers on day 17 of the estrous cycle (cyclic; n = 4) and day 17 of pregnancy (pregnant; n = 4). Total RNA (200 ng/ml) underwent Solexa 50 nt single-end sequencing using the Illumina HiSeq 2000 sequencer. Full-length small RNA tags were then mapped to the bovine genome (UMD3.1) using the short oligonucleotide alignment program (SOAP) to analyze their expression and distribution. Only reads that mapped to the genome were aligned in miRBase (Release 19) to miRNA precursors with no mismatch, and to the mature miRNA. Unannotated small RNAs were used to predict novel miRNAs using Mireap prediction software (http://sourceforge.net/projects/mireap/). As a result, 85% of the annotated small RNA were identified as miRNA, 2% mapped to introns, exons, repeats or other types of small RNA, and the remaining 13% were unannotated, and therefore potentially novel miRNAs. The total number of annotated miRNA expressed in the CL was 544, of which ~40% had low reads (<5 reads), ~50% had reads between 5 and 4500, and ~10% had reads above 4500. Differential expression analysis using DEseq resulted in 12 miRNA that were significantly different ($P_{adj} < 0.05$). Eight miRNA (miR222, miR345-3p, miR345-5p, miR541, miR1307, miR2389, miR2443, miR6517) were downregulated and four miRNA (miR1-1, miR10b, miR122 and miR302b) were upregulated in the CL of pregnancy. Using TargetScan, we determined 2431 predicted target mRNA, 172 predicted genes to be targeted by at least 3 or more of the differentially expressed miRNA. Further analysis of these common targets for Gene Ontology (GO) demonstrated all these genes to be enriched for apoptosis and immune response signaling pathways. In addition, 226 unannotated small RNAs were identified as novel miRNA that have not been reported for any species, with 46 of these being expressed in at least 3 of the 4 treatment replicates. Three of the novel miRNA (bta-miR10163, bta-miR10178, bta-miR10170) were differentially expressed in the CL of cyclic vs pregnant cattle. GO analysis of these 3 miRNA also revealed predicted functions of regulation of apoptosis and T cell functions, as well as cellular biosynthetic pathways and metabolism. These data confirm the differential expression of miRNA in the CL during maternal recognition of pregnancy, and highlight a potential role for miRNA in modulating the immune response to rescue the CL. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2012-67015-30212 from the USDA National Institute of Food and Agriculture.

Objective B: Identify inter-cellular interactions between somatic cells of the ovary, somatic cells and germ cells, or somatic cells and the embryo that promote follicular growth, oocyte maturation, early embryonic development, and establishment and maintenance of pregnancy.

Our *working hypothesis* is that bi-directional communication between somatic and germ cells of the ovary as well as between immune cells and components of the female reproductive tract is essential for the production of developmentally competent oocytes, maintenance of the CL, and implantation.

A. Role of leukocyte adhesion molecules in recruitment of immune cells into the CL. L Steinberger, J Pate, D Townson (NH)

Hypothesis: Leukocyte adhesion molecules expressed on luteal endothelial cells vary with functional state of the CL, facilitating infiltration of immune cells only after the CL is fully functional. The

objective of this experiment was to determine if prostaglandins alter adhesion molecule expression on endothelial cells of midcycle corpora lutea (days 12-15) compared to early corpora lutea (days 5-7) to allow for immune cell infiltration into the tissue. Luteal endothelial cells (LEC) were isolated from early and midcycle corpora lutea by size exclusion. Endothelial cells were cultured in RPMI + 20% fetal bovine serum (FBS) + gentamicin in 6 well plates for 72 hours to allow for cell attachment. After 72 hours, cells were washed and cultured for another 24-48 hours to allow for cell proliferation. When cells reached ~80% confluency, they were washed and cultured in RPMI + gentamicin + ITS overnight. Cells were treated with a physiological (10 ng/ml) or supraphysiological (1□M, which was 353-476 ng/ml, depending on the prostaglandin) concentration of each prostaglandin for 24 hours. Cells were collected and analyzed for concentration of vascular cell adhesion molecule 1 (VCAM1), selectin P (SELP) and chemokine (C-C motif) ligand 2 (CCL2) mRNA by qPCR. Additionally, LEC expression of prostaglandin receptor mRNA was determined by PCR. Tumor necrosis factor 1 (TNF; 10 ng/mL) and no treatment were used as controls.

PGE_2 (PTEGR2, PTEGR4), $PGF_{2\alpha}$ (PTGFR) and PGI_2 (PTGIR) receptor mRNA were expressed by LEC. Concentration of CCL2 mRNA was reduced by both physiological and supraphysiological concentrations of $PGF_{2\alpha}$ in early LEC and by PGI_2 in midcycle LEC. Both concentrations of PGE_2 reduced CCL2 in early and midcycle CL. VCAM1 was reduced by both concentrations of PGI_2 in midcycle LEC only. Samples were analyzed for differences between low and high concentrations of prostaglandins. There was a significant difference between the two concentrations of PGE_2 in the concentration of VCAM1 in midcycle LEC and in SELP concentration in early LEC. There was a tendency for a difference between concentrations of PGI_2 in SELP concentration in early LEC. This is important because many studies have used 1 μM of prostaglandin, which is supraphysiological. In this study, effects on some mRNA were exaggerated by the supraphysiological concentration of PG.

TNF treatment upregulated the expression of CCL2 and VCAM1 by LEC isolated from early and midcycle luteal tissue and SELP by midcycle LEC. Previous reports have shown that CCL2 is upregulated by TNF (50-100 ng/mL) in CLENDOs of early pregnancy. However, these are the first data to demonstrate increased SELP and VCAM1 mRNA in response to TNF in LEC.

B. Effect of Bovine Luteal Cell-derived Exosomes on Immune Cells. Katie Branham, Troy Ott and Joy Pate

The corpus luteum (CL) is essential for production of P_4 and maintenance of pregnancy in mammals. Immune cells infiltrate the CL, but the role of immune cells in the fully functional CL has not been elucidated. Luteal cell communication with T lymphocytes (T cells) can program the T cell responses, and luteal cells from fully functional CL can activate T cells through paracrine mediators. We hypothesized that luteal cells secrete extracellular vesicles, such as exosomes, which may serve as a means of communication from luteal steroidogenic cells to resident immune cells. The objectives of this study were to determine if the physiological state of the CL influences the quantity of secreted exosomes, if luteal cell-derived exosomes can activate T cells, and if luteal cell-derived exosomes can drive macrophage polarization. All experiments were repeated using CL from a minimum of 4 different animals. Exosomes were isolated from midcycle and regressing bovine luteal cells and were quantified and evaluated using NanoSight and NanoSight Tracking Analysis (NTA). Carboxyfluorescein succinimidyl ester (CFSE)-labeled exosomes were cultured with anti-CD3-labeled T cells and their uptake was visualized using a FlowSight visualizing flow cytometer. Next, midcycle

luteal cell-derived exosomes were cultured with CFSE-labeled T cells and proliferation was measured by flow cytometry. Finally, midcycle luteal cell-derived exosomes were added to undifferentiated monocytes and expression of macrophage cell type-specific genes was determined using quantitative polymerase chain reaction (qPCR). The mRNAs quantified were tumor necrosis factor (TNF), interleukin 1-beta (IL1B), and inducible nitric oxide synthase (NOS2) to identify classically-activated macrophages, and cluster of differentiation 36 (CD36), interleukin 10 (IL-10), and cluster of differentiation 163 (CD163) to identify alternatively activated macrophages. Bovine luteal cells from both physiological states released exosomes into culture media. Luteal cells from regressing CL released fewer ($P<0.05$) exosomes than midcycle luteal cells. Exosomes were internalized into T cells, and midcycle luteal cell-derived exosomes induced T cell proliferation. Midcycle luteal cell-derived exosomes induced macrophages that were characterized by highly significant expression of TNF, IL1B and NOS2 mRNA. When monocytes were primed toward an alternative differentiation pathway, luteal cell-derived exosomes increased ($P<0.001$) expression of CD163 and CD36. In conclusion, we have demonstrated that bovine luteal cells release exosomes that may facilitate communication with immune cells. This provides an alternative method to direct cell-cell contact by which luteal cells communicate with immune cells to control the luteal microenvironment.

C. Zinc is involved in progesterone production. Francisco Diaz

Progesterone is an essential hormone involved in the function of a variety of reproductive tissues including the ovary, uterus and mammary gland. Progesterone production is upregulated in granulosa cells (cumulus and mural) after the LH surge, but the intra-follicular mechanisms regulating this transition are not completely known. Recent findings show that zinc is an essential factor for ovarian function including oocyte maturation, cumulus expansion, and follicle rupture. In this study, we provide evidence that zinc is involved in a pathway that inhibits progesterone production. The findings show that a zinc chelator, TPEN, increases expression of *Cyp11a1* and *Star* mRNA between 8-20 fold and progesterone production more than 3 fold in cultured COC. Feeding a zinc deficient diet for 10 days, but not 3 days caused a ~2.5 fold increase in *Star*, *Hsd3b2* and *Ptgfr* mRNA. Progesterone is important for oocyte developmental potential because treatment of COC with the progesterone synthesis inhibitor, aminoglutethimide, reduced blastocyst formation from 79% to 48%. In vivo, feeding a zinc deficient diet for 5 days before ovulation did not affect number of CL present on the ovary, STAR protein or serum progesterone, but did result in a 6 fold increase in *Hsd3b2* and 2 fold increase in *Ptgfr* mRNA. More significantly, incubating luteal tissue from a mid-cycle CL with TPEN increased 2-3 fold expression of *Star*, *Hsd3b2* and *Ptgfr* mRNA and increased 3 fold the amount of progesterone that accumulated in the media. Collectively, the results show that zinc acutely suppresses steroidogenic transcripts and in vitro accumulation of progesterone in the medium following culture of COC or luteal tissue.

D. Induction of tolerogenic mediators in uterine immune cells during early pregnancy in dairy heifers. MM Kamat, S Vasudevan, JL Pate, TL Ott

Problem: Embryo loss during early pregnancy contributes to infertility. A portion of these losses are hypothesized to be immune mediated but little is known about the immune cell responses to the presence of the embryo. This study tested the hypothesis that endometrial resident immune cells are induced to a tolerogenic phenotype by signals emanating from the developing conceptus.

Method of Study: Uterine tissue was collected from Holstein dairy heifers on Day 17 of the estrous cycle and Days 17 and 20 of early pregnancy. Tissues were labeled with antibodies against CD47 and Indoleamine 2,3-dioxygenase (IDO) and labeling intensity was analyzed using ImageJ software (n=5 heifers/status/Day). In addition, abundance of mRNA for CD172a and CD47 were analyzed in RNA extracted from whole endometrium by quantitative PCR (n=7-9 Day 17 cyclic, n=6 Day 17 pregnant and n=5 Day 20 pregnant). Results were analyzed using MIXED procedures of SAS using preplanned orthogonal comparisons.

Results: Endometrial labeling intensity for CD47 was greater ($p=0.05$) in endometrium from pregnant compared to cyclic heifers. Differences in labeling intensity were greatest in the shallow stroma and shallow glands ($p<0.05$). Labeling intensity for IDO staining was greater ($p<0.05$) in endometrium from Day17 pregnant heifers compared to Day17 cyclic heifers. Among pregnant heifers, labeling intensity was almost 3 fold greater at Day 17 versus Day 20 ($p<0.01$). Differences in IDO labeling intensity were greatest in the luminal epithelium, but were also detected in the shallow glands, deep glands and myometrium. No differences were observed in total endometrial mRNA abundance for CD47 and CD172a.

Conclusions: Results presented here support the hypothesis that conceptus signals affect resident immune cells at very early stages of pregnancy. We hypothesize that increased CD47 expression interacts with its receptor, CD172a, to induce inhibitory signals via Immunoreceptor Tyrosine based Inhibitory Motifs (ITIM). This interaction is known to bring about cell-cell adhesion and T cell inactivation. Increased IDO expression may induce production of kynurenine, a ligand for the Aryl Hydrocarbon Receptor (AhR) and is involved in Regulatory T cell generation. Thus, during early pregnancy in cattle, embryonic signals, including IFN tau, may promote development of tolerogenic immune phenotype for successful establishment of pregnancy.

E. Effects of early pregnancy on endometrial immune cell phenotype and function in dairy heifers. Ott, TL, Kamat, MM, Vasudevan, S, Hartzell, MC and Pate, JL

Problem: Infertility and subfertility are issues relevant to humans, domesticated animals and endangered species. Pregnancy in ruminants is established in an environment of transient secretion of a unique, type I interferon, interferon tau (IFNT). IFNT rescues the corpus luteum (CL) through local actions on the endometrium and also initiates a cascade of expression of interferon-stimulated genes across the entire uterine wall and extending to circulating immune cells and the CL. Our working hypothesis is that pregnancy and IFNT induce changes in immune cells that promote tolerance, tissue remodeling and angiogenesis.

Method of Study: Twenty dairy heifers were randomly allocated to Day 17 cyclic (n=5-9), Day 17 pregnant (n=5-7) and Day 20 pregnant (n=4-5) treatment groups and uteri, lymph node, spleen and blood were collected at slaughter. Flow cytometry, immunofluorescence (IF) labeling and qPCR were used to determine effects of pregnancy on endometrial immune cells expressing markers for natural killer (NK) and cytotoxic ($CD8^+$) T cells and for macrophages/dendritic cells. In addition, mRNA abundance of pro-inflammatory (Th1) and anti-inflammatory (Th2) cytokines and other regulatory molecules was determined in the endometrium.

Results: Early pregnancy was accompanied by a marked increase ($P<0.05$) in the proportion of endometrial $CD45^+$ cells expressing markers for natural killer (NK) cells and cytotoxic T cells (CD8) and an increase ($P<0.05$) in $MHCII^+$ cells, particularly around shallow glands of the endometrium of pregnant heifers on Day 20. Quantitative PCR analysis showed increased abundance of mRNA for interleukin (IL)-15, an NK cell growth factor, and IL-10, a tolerogenic cytokine, in the endometrium during early pregnancy. Furthermore, expression of indoleamine 2,3 dioxygenase (IDO) was ~15 fold greater in pregnant compared to cyclic heifers at Day 17, but then IDO mRNA and protein abundance declined by Day 20 of pregnancy to amounts similar to Day 17 cyclic heifers. IDO converts tryptophan to kynurenine, which is thought to alter immune function by activation of the aryl hydrocarbon receptor (AHR). Endometrial expression of the AHR was detected, but there were no effects of day or pregnancy status. Pregnancy induced expression of CD172a, a marker for immature dendritic cells, and for inhibitory proteins PDL1 (Status * Day; $P<0.05$), LAG3 (Status; $P=.06$) and CTLA4 (Status; $P<0.05$). It is interesting that expression of IDO, IL10 and several other immune cell regulators decreased between Day 17 and 20 of pregnancy, suggesting a strong induction and then repression of selected responses.

Conclusions: Overall, a picture is emerging that early pregnancy signaling at the fetal-maternal interface involves immune cell activation as well as induction of key regulatory molecules known to mediate tolerance. We show here a pattern of induction and then repression of key mediators of immune function, which we postulate serves as a developmental switch to promote an immune privileged niche and tissue remodeling in the endometrium during early pregnancy. These results should help better define the mechanisms that promote establishment of pregnancy and formation of the placenta in ruminants.

F. Vitamin D and vitamin D receptor signaling effects on implantation and maternal T cells and NK cells during murine pregnancy. Lindsay Snyder, Manasi Kamat, Sreelakshmi Vasudevan, James Hester, Alison Gernand, Francisco Diaz, Troy Ott, Margherita Cantorna

Problem: Maternal vitamin D deficiency has been linked to increased rates of miscarriage, pre-ecclampsia, pre-term birth, and low birth weight. Vitamin D is also a potent immunomodulator and immune cells, including regulatory T cells and NK cells, are known to influence pregnancy outcomes; thus we hypothesize the existence of an immunological link between maternal vitamin D status and reproductive outcomes.

Methods of Study: Vitamin D sufficient (+D; n=6), severely vitamin D deficient (-D; n=5), and vitamin D receptor knockout (KO; n=4) mice were bred and weaned onto purified +D/-D diets for five weeks. At eight weeks of age, they were paired with BALB/c wild type males to generate allogeneic timed pregnancies. At gestational day (Gd) 12.5, serum, reproductive tracts, and spleens were collected. Serum was used to measure 25D3 levels to confirm vitamin D status. Implantation sites were enumerated and spleens were utilized for flow cytometry to characterize regulatory T cell (CD3+CD4+FoxP3+CD25+) and NK cell (CD3-NKp46+) populations.

Results: +D mice had higher serum 25D3 concentrations than –D mice (68.69 ng/ml vs 18.90 ng/ml, $p=0.01$). At Gd 12.5, -D mice had fewer implantations than +D mice (6.20 vs 8.33; $p=0.04$) and surprisingly, VDR KO mice tended to have more implantations than +D mice (10.25 vs 8.33, $p=0.17$). Additionally, after detection of vaginal mucus plugs, pregnancy rates were 52% for +D mice, 42% for -

D mice, and 73% for KO mice. The only immunological changes detected were in the KO mice. They tended to have elevated splenic regulatory T cell frequencies compared to the +D and -D mice (15.53 vs 12.58 and 11.70, p=0.06).

Conclusion: Vitamin D deficiency decreased implantation success. However, the absence of VDR signaling tended to enhance implantation success and regulatory T cell numbers. Changes in immune cell proportions and function may explain some of the differences we observed; however, ovulation rates may also be a contributing factor. In conclusion, vitamin D and VDR signaling affected implantation and regulatory T cell proportions. This supports our hypothesis that vitamin D and VDR signaling effects on pregnancy outcome may be mediated via modulation of maternal immunity.

G. Prepregnancy maternal vitamin D deficiency and placental development in mice. Alison D. Gernand, Margherita T. Cantorna, Francisco J. Diaz, Lindsay M. Snyder, James M. Hester, Sreelakshmi Vasudevan, Kamat Manasi Manohar, Troy L. Ott

Problem: Maternal vitamin D deficiency is a public health problem worldwide and is linked to numerous adverse pregnancy outcomes with placental origins, including fetal growth restriction and preeclampsia. Our objective was to examine the effect of prepregnancy vitamin D deficiency on placental vascular development.

Method of Study: We modeled vitamin D deficiency using a Cyp27B1 genetic knockout (KO) mouse with no dietary vitamin D (n=6); wild-type (WT) mice with a vitamin D sufficient diet were the control (n=6). All mice were fed the respective diet from 3 weeks of age onward. At 8 weeks of age, mice were mated to genetically dissimilar males to produce allogeneic embryos. Mice were euthanized at 12.5 days gestation. We measured morphometry of uterine horns, placentas, and embryos. In placentas, mRNA was estimated by qPCR for angiogenic proteins (angiopoeitin-1 (Ang-1), Ang-2, vascular endothelial growth factor (VEGF), placental growth factor (PlGF)).

Results: KO mice had fewer fetuses at 12.5 days compared to WT (6.2 vs. 8.5, p=0.033). There were no statistically significant differences for KO vs. WT in uterine horn, placental, or fetal weights or Ang-1, Ang-2, or PlGF gene expression (all p>0.05). However, KO placentas exhibited significantly decreased VEGF expression compared to WT placentas (p=0.047, Figure 1). KO mice seemed less likely to become pregnant after mating, but this was not tested due to the small sample size.

Conclusions: Prepregnancy maternal vitamin D deficiency in mice caused lower litter size among mice that became pregnancy and decreased VEGF expression. Future work should examine the mechanisms for these effects and prepregnancy vitamin D status and placental development in women.

Accomplishments – Major findings, Key Outcomes

1. Changes in microRNA expression in the CL may facilitate luteal rescue during maternal recognition of pregnancy. The miRNA that change during MRP target pathways involved in apoptosis and immune response.
2. Luteal cell-derived extracellular vesicles

3. Acute zinc depletion promotes progesterone accumulation in media from cumulus cells or luteal tissue cultured in vitro.
4. During early pregnancy in cattle, increased CD47 expression induces inhibitory signals known to bring about cell-cell adhesion and T cell inactivation.
5. Increased IDO expression induce production of kynurenine, a ligand for the Aryl Hydrocarbon Receptor (AhR) that is involved in regulatory T cell generation.
6. During early pregnancy in cattle, embryonic signals, including IFN tau, may promote induction and then repression of key mediators of immune function, which we postulate serves as a developmental switch to promote an immune privileged niche and tissue remodeling in the endometrium during early pregnancy.
7. Vitamin D may affect implantation success via modulation of maternal immunity.

Opportunities for training and professional development

Graduate and undergraduate students have developed their skills in development and testing of hypotheses, experimental design and analysis of data. They were taught all of the procedures and assays necessary to perform these experiments. Further professional development included the opportunity to present their results at annual meetings of the Society for the Study of Reproduction and the American Society of Reproductive Immunology.

How results have been disseminated to target audience

Articles in scientific journals, presentations at national and international meetings and to producers and veterinarians

Plan for next reporting period

- Studies will continue on the type and role of macrophages in the CL (PA, WI)
- Studies will continue on immune cell regulation of luteal function, including the role of endothelial cells (PA, NH)
- Work will continue on the role of zinc in ovarian function
- Studies will quantify effects of conceptus signals on immune cell phenotype and function during early pregnancy in dairy cows (PA)
- Studies will quantify effects of early pregnancy on expression of interferon stimulated genes in peripheral blood leukocytes during early pregnancy in dairy cows (PA, NY)
- Studies will determine role of Vitamin D in vascular development of the placenta (PA)

Major Changes

Support

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- National institute of health research grants HD074831 to FJD.

- Agriculture and Food Research Initiative Competitive Grant no. 2012-67015-30212 from the USDA National Institute of Food and Agriculture to JLP.
- C. Lee Rumberger and Family Chair in Agricultural Science funds to JLP

ANNUAL REPORT OF COOPERATIVE REGIONAL RESEARCH PROJECT NE-1227

Virginia Polytechnic Institute and State University
Department of Animal and Poultry Sciences
2014 Annual Report

Project: NE-1227, Ovarian Influences on Reproductive Success in Ruminants

***Participants (FTE)*:** Virginia Polytechnic Institute and State University, Department of Animal and Poultry Sciences, Principal Leader: Michelle Rhoads (0.2); Graduate Students: Abby Zezeski (0.5), Vicki McCracken (0.1), Jeff Wiegert (0.1), Rebecca Poole (0.5); Post-doctoral Researcher: Abdullah Al Naib (0.5); Primary Collaborators: Alan Ealy (0.2), Kiho Lee (0.2)

***Target Audience*:**

Target Audiences: Scientists, students and trainees working in similar areas, livestock producers and other stakeholders.

Efforts: Peer-reviewed abstracts and publications, conference presentations, formal classroom instruction, laboratory instruction, experiential learning for graduate and undergraduate students through research opportunities.

Products:

Journal Articles:

*Brown, B.M., J.W. Stallings, J.S. Clay and **M.L. Rhoads**. 2015. Milk production and composition of dairy cows that were exposed to heat stress at or around the time they were conceived. PLoS One. (in review).

Rhoads, M.L., A.L. Zezeski, V.L. McCracken, G.A. Perry and A.D. Ealy. 2015. Maturation of bovine cumulus-oocyte complexes in varying concentrations of follicular fluid improves cumulus cell expansion without affecting the outcome of in vitro fertilization. Reprod. Fertil. Dev. (in review).

*Brown, B.M., J.W. Stallings, J.S. Clay and **M.L. Rhoads**. 2015. Periconceptional heat stress of Holstein dams is associated with differences in daughter milk production during their first lactation. PLoS One. (in review).

*McCracken, V.L., G. Xie, S.E. Deaver, L.H. Baumgard, R.P. Rhoads and **M.L. Rhoads**. 2015. Hepatic progesterone-metabolizing enzymes cytochrome P450 2C and 3A in lactating cows during thermoneutral and heat stress conditions. J. Dairy Sci. (in press).

*Deaver, S.E., A.M. Felix and **M.L. Rhoads**. 2015. Reproductive performance of lactating dairy cattle after intrauterine administration of a prostaglandin F2α receptor antagonist four days after insemination. Theriogenology. 83(4):560-566.

Conference Proceedings:

Rhoads, M.L. 2014. Implementing strategies to reduce heat stress in dairy herds. Proc. ReprodAction Cattle Symp. 4-8.

Abstracts:

Al Naib*, A., A.Y. Wood, H.L.M. Tucker, C.M. Parsons, V.L. McCracken, A.L. Zezeski, S.E. Deaver, B.M. Brown, R.M. Akers and **M.L. Rhoads**. 2015. Effects of Tamoxifen on pre-pubertal heifer reproductive tissues: Potential for disruption of tract development through alteration of related signaling pathways. J. Dairy Sci. (in press)

Wiegert, J.G.*, R.H. Preisser, M.C. Lucy, T.J. Safranski, R.P. Rhoads, J.W. Ross, L.H. Baumgard, M.J. Estienne and **M.L. Rhoads**. 2015. Effects of in utero heat stress on subsequent lactational performance of gilts and transgenerational effects on offspring. J. Anim. Sci. 93(suppl 2):166.

Safranski, T.*, M.C. Lucy, J.N. Rhoades, M. Estienne, J.G. Wiegert, **M. Rhoads**, R.P. Rhoads, L.H. Baumgard and J.W. Ross. 2015. Reproductive performance of gilts having developed in heat stressed dams. J. Anim. Sci. 93(suppl 2):85.

McCracken, V.L.*, A.Y. Wood, A.L. Zezeski, J.G. Wiegert, D.W. Lugar, J.M. Scheffler, S.W. El-Kadi, A.D. Ealy and **M.L. Rhoads**. 2014. Porcine pre-pubertal reproductive tract development is altered by consumption of high fructose but not high fat diets. Biol. Reprod. (in press).

Invited Presentations:

Periconceptional heat stress of Holstein dams is associated with differences in daughter milk production and composition.
Iowa State University Symposium on Climate Change: Biological Consequences of Heat Stress.
April 2015

Periconceptional heat stress of Holstein dams affects daughter milk production and composition.
Roy A. Wallace Memorial Symposium on Bovine Reproduction, Select Sires Inc.
October 2014

Implementing strategies to reduce heat stress in dairy herds.
Ceva Sante Animale ReprodAction Cattle Symposium
Nice, France
March 2014

Other Products:

Accomplishments – Major findings, Key Outcomes

Objective A: Identify intracellular signaling pathways and gene expression regulatory mechanisms within the ovary, embryo, or female reproductive tract that promote oocyte growth and maturation, fertilization, early embryonic development, and establishment and maintenance of pregnancy

Effects of Tamoxifen on pre-pubertal heifer reproductive tissues: Potential for disruption of tract development through alteration of related signaling pathways.

Al Naib, A., A.Y. Wood, H.L.M. Tucker, C.M. Parsons, V.L. McCracken, A.L. Zezeski, S.E. Deaver, B.M. Brown, R.M. Akers, and M.L. Rhoads

Pre-pubertal exposure of the developing reproductive tract (RT) to estrogen or xenoestrogens can have long-term consequences that compromise the reproductive performance of dairy cattle. This research examined the effects of the selective estrogen receptor modulator, tamoxifen (TAM), on gene and protein expression in the pre-pubertal RT with particular focus on signaling pathways that affect RT morphology. Tamoxifen was administered to heifer calves (n=7) daily (0.3 mg/kg subcutaneously) from 28 to 120 d of age. Control calves (CON; n=7) received an equal volume of excipient. Weight, gross measurements and samples of RT tissues were collected upon sacrifice at 120.7 ± 0.3 d of age. Protein and mRNA were extracted from snap frozen samples of vagina, cervix, uterine body, ovary and oviduct. As we previously reported, overall weight of the RT was dramatically decreased following TAM treatment (P=0.01). Both estrogen receptor alpha (ERα) protein and gene expression were dramatically reduced in the uterus, cervix and vagina (P<0.01). In oviduct, ERα protein was reduced in the TAM treated animals (P<0.01) while ERα gene expression was not affected. Similarly, the phosphorylated form of ERα protein was reduced in the cervix and vagina (P<0.01) and tended to be reduced in the uterus (P=0.09) of the TAM group. In contrast to other RT tissues, phosphorylated ERα protein abundance was greater in the ovaries of the TAM animals (P<0.05). Even though insulin-like growth factor-I (IGFI) gene expression was higher in the uterus, oviduct and vagina of the TAM group (P<0.01), IGFI receptor protein and gene expression did not differ. Total mitogen-activated protein kinases (MAPK) protein was higher in the oviduct, vagina and ovary (P<0.01) but was lower in the uterus of TAM treated heifers (P<0.01). The phosphorylated form of MAPK protein was similarly increased in the ovary but was decreased in the cervix of the TAM group (P<0.05). In conclusion, the bovine pre-pubertal RT is affected by TAM treatment. Further research is needed to determine if these effects have long-term consequences for reproductive performance.

Porcine pre-pubertal reproductive tract development is altered by consumption of high fructose but not high fat diets.

V.L. McCracken1, A.Y. Wood1, A. L. Zezeski1, J.G. Wiegert1, D.W. Lugar1, J.M. Scheffler1, S.W. El-Kadi1, A.D. Ealy1, and M.L. Rhoads1

Pre-pubertal maturation of the female reproductive tract has traditionally been linked with whole-body adiposity. Previous work from our laboratory suggested that inclusion of sugars in diets fed to gilts accelerated reproductive tract development independent from body adiposity. Because of these findings, the current study was designed to more clearly delineate the direct effects of fat and/or sugar intake on the maturation of the pre-pubertal reproductive tract. Three week old gilts (n=40) were placed on one of five dietary treatments: 15% fat included, 35% fructose included, both fat and fructose included, and two different control diets. The first control diet was a standard industry diet meant to result in optimal lean growth. The second control diet was designed to account for reduced feed intake (and therefore, reduced lysine intake) by the gilts receiving diets containing fat. Thus, this

second control diet was fed to match the lysine intake of the gilts receiving fat-containing diets. Gilts were penned in pairs within treatments in a single barn with 11 h/13 h day/night cycle. The treatment groups that received the fat and fat + fructose diets were fed for ad libitum intake. The fructose and lysine-restricted groups were pair fed to match the intake of the fat and fat + fructose groups. Each week, body weight and back fat (via ultrasonography) measurements were recorded. On week nine of the trial, one gilt from each pen (n=4/treatment) was scanned with dual-energy X-ray absorptiometry to determine final body adiposity and then sacrificed for collection of reproductive organs. Visible antral follicles were counted on each ovary and the tract was weighed. Portions of the reproductive tract were then quickly preserved for further analyses while other portions were retained for additional measurements such as volume (left ovaries only) and length (left uterine horns only). Body weights did not differ amongst any of the 5 treatments on the day of sacrifice ($p=0.32$). Differences in body adiposity were solely based on whether or not the gilts consumed high levels of dietary fat (16.8±0.7 v. 8.5±0.5% for fat and non-fat diets, respectively; $p<0.01$). Visible follicle counts (32.4±6.0 v. 16.0±5.0; $p=0.05$), ovary volume (103.7±16.3 v. 52.9±15.3 mm3/kg of body weight; $p=0.04$) and uterine horn length (9.18±0.58 v. 7.40±0.54 mm/kg of body weight; $p=0.04$) were greater in gilts consuming fructose (fructose and fat + fructose diets) compared to those that were not, but these variables were not affected by fat consumption. As a percentage of body weight, total reproductive tracts were heavier in gilts consuming fructose-containing diets compared to non-fructose diets (1.3±0.1 v. 0.8±0.1%; $p=0.01$). Individually, cervical (0.28±0.04 v. 0.13±0.04%; $p=0.01$), uterine (0.91±0.08 v. 0.65±0.07%; $p=0.03$) and total ovarian weights (0.10±0.01 v. 0.06±0.01%; $p=0.04$) were greater in fructose fed gilts compared to non-fructose treatments. Taken together, these results suggest that fructose consumption increases reproductive tract size and advances ovarian parameters of impending puberty regardless of fat consumption and body adiposity. These results demonstrate the sensitivity of the pre-pubertal female reproductive tract to sugar consumption. Future analyses will determine the long-term consequences of high sugar intake for fertility.

Objective B: Identify inter-cellular interactions between somatic cells of the ovary, somatic cells and germ cells, or somatic cells and the embryo that promote follicular growth, oocyte maturation, early embryonic development, and establishment and maintenance of pregnancy.

Maturation of bovine cumulus oocyte complexes in media containing follicular fluid improves cumulus cell expansion without affecting the outcome of in vitro fertilization

Wood, A.Y., A.L. Zezeski, V.L. McCracken, A.D. Ealy, M.L. Rhoads

The magnitude of cumulus cell expansion during the maturation phase affects the ability of bovine oocytes to undergo fertilization in vitro and subsequent embryo competency. The objective of this study was to determine whether inclusion of follicular fluid in maturation medium would improve cumulus cell expansion and in vitro fertilization rates. In the first study, follicle fluid was added to a base maturation medium (Medium 199 with 10% fetal bovine serum and 0.2% follicle stimulating hormone) at rates that resulted in media containing 0, 25, 50, 75 and 100% follicle fluid. Cumulus oocyte complexes (COCs; n=877, 129-176 per treatment over 14 replicates) aspirated from slaughterhouse-derived ovaries were randomly assigned to treatments and cultured individually in 10 µl drops on untreated 100 mm plates covered in mineral oil. Images of each individual COC were captured at 0, 6, 12 and 19 hours relative to placement into the maturation medium and follicle fluid

treatment. Images of the COCs were analyzed with ImageJ to calculate total area (μm2). After 6 hours in culture, expansion of the COCs in 75% follicle fluid was greater than those in 0 ($P<0.01$), 25 and 100% follicle fluid ($P<0.05$), but was similar to those COCs cultured in 50% follicle fluid ($P=0.13$). At the 12 hour and 19 hour time points, COCs in 75% follicle fluid expanded more than all treatments (0, 25 and 100% $P<0.01$, 50% $P<0.05$), whereas those in 50% follicle fluid had expanded more than 0 and 25% follicle fluid ($P<0.05$). Overall percent increase from hour 0 to hour 19 was 252.61±9.76% for COCs in 75% follicle fluid, which was significantly greater than all other treatments ($P<0.01$). Those COCs cultured in 50% follicle fluid increased 192.54±10.21%, which was greater than 0% ($P<0.05$) and 25% ($P<0.01$). Because the greatest amount of cumulus cell expansion was observed for COCs matured in 75% follicle fluid, a second study was conducted during which in vitro fertilization was performed on COCs matured in either 0% or 75% follicle fluid (n=37-160 per treatment per replicate, 10 replicates). Cumulus oocyte complexes matured in their assigned treatment groups either for 12 or 19 hours, were then fertilized in groups of 50 COCs per 500 μl of fertilization medium with a mixture of semen from two Angus bulls that were gradient-purified. When comparing 0% and 75% follicle fluid, no difference was seen in day 3 cleavage rates (46.23±4.95% vs. 48.81±4.95%) or day 8 blastocyst rates (16.58±3.87% vs. 9.49±3.87%). Regardless of follicle fluid treatment, maturation for 19 hour maturation compared to 12 hour maturation resulted in greater day 3 cleavage rates (56.96±4.43% vs. 38.08±5.42%; $P<0.05$) and day 8 blastocyst rates (18.98±3.47% vs. 7.10±4.25%; $P<0.05$). In summary, maturation of COCs in 75% follicle fluid resulted in the greatest amount of cumulus cell expansion during in vitro maturation. However, this hastening in cumulus expansion did not impact the fertilization nor the developmental potential of oocytes when fertilized after shortened (12 hours) or normal maturation intervals (19 hours).

Opportunities for training and professional development

Graduate and undergraduate students were thoroughly trained in whole animal and molecular techniques. Some of these include transvaginal follicle aspiration, post-mortem tissue collections, histological analyses, mRNA extraction and assessment and radioimmunoassay measurement of circulating hormones. All have developed their skills in development and testing of hypotheses, experimental design and analysis of data. Both graduate and undergraduate students were presented with opportunities to report their results at local and national meetings.

How results have been disseminated to target audience

Results have been disseminated through written means including publication of abstracts, manuscripts and conference publications. Results have also been disseminated orally through presentations and classes led by the project leader, collaborators, graduate students and undergraduate students.

Plan for next reporting period

- We will continue to investigate the molecular aspects of the communication between the cumulus cells and the oocyte to determine how aberrations affect fertility.
- Additional studies investigating the effects of fat/sugar intake on puberty and subsequent fertility are on-going.
- Analyses of the ultrastructure of oocytes exposed to high or low levels of estradiol at estrus are on-going.

- Studies investigating the relative contribution of the oocyte vs. cumulus cells to the heat stress response are being organized.

Major Changes

None

Support

In addition to Hatch Multistate Funds, these studies were supported by:

- National Pork Board: Impact of in utero heat stress on subsequent lactational performance and performance of offspring.
- Virginia Horse Industry Board: Therapeutic effect of resveratrol on metabolism and reproductive parameters in overweight mares.
- John Lee Pratt Animal Nutrition Program: Effects of high-energy diets on metabolic and reproductive parameters of growing female pigs.
- John Lee Pratt Animal Nutrition Program: Grape seed extract as a natural antimicrobial and antidiarrheal agent to replace antibiotics and zinc oxide in commercial pig feed.

ANNUAL REPORT OF COOPERATIVE REGIONAL RESEARCH PROJECT NE-1227

KENTUCKY AGRICULTURE EXPERIMENT STATION

1. Project NE-1227; Ovarian Influences on Reproductive Success in Ruminants

2. Cooperating Agencies and Principal Leaders:

- University of Kentucky; Department of Animal and Food Sciences: P.J. Bridges

3. Participants:
- Phillip Bridges (0.8 FTE)
- Katheryn Cerny (0.5 FTE)

Non-Technical Summary

At a recent workshop, a group of more than 75 stakeholder scientists from federal, public, and private institutions across the United States recommended the following high impact areas as funding priorities for USDA/CSREES: development of the oocyte, follicle recruitment and development, identifying genes involved in gamete quality and embryo development, uterine conceptus interactions with emphasis on embryonic and fetal survival, reproductive immunology, and gonadal development. Our stakeholders include animal producers, the scientific community, and citizens of the region and the nation. By enhancing basic knowledge of the underlying biology surrounding ovarian function and embryonic survival, new strategies can be developed for application by producers and veterinarians. Application of management strategies that are not based upon drug development or use of new drugs is economical, user and consumer friendly, and preserves food quality and safety.

Accomplishments

Major goals of the project

Impaired reproductive performance is a major cause of reduced productivity for ruminants and of reduced profitability for dairy and meat animal producers. The focus of the NE-1027 Regional Project (and its predecessor, the NE-1007, NE-161 Regional Project) has been to address nutritional, management, and environmental factors that impact ovarian activity and subsequent pregnancy rates in domestic ruminants. Our current goal is to continue this important investigative work, focusing on inter and intra-cellular communication mechanisms that regulate oocyte growth and maturation, corpus luteum (CL) development, maintenance and regression, and early embryonic development. Alterations in inter- and intra-cellular communication due to metabolic and/or environmental stress will also be studied. Our current objectives are 1) to identify intracellular signaling pathways and gene expression regulatory mechanisms within the ovary, embryo, or female reproductive tract that promote oocyte growth and maturation, fertilization, early embryonic development, and establishment and maintenance of pregnancy; and 2)to identify inter-cellular interactions between somatic cells of the ovary, somatic cells and germ cells, or somatic cells and the embryo that promote follicular growth, oocyte maturation, early embryonic development, and establishment and maintenance of pregnancy. Participation from Kentucky (how does the ovary regulate oviductal function and early embryonic development) lies under objective 1.

ANNUAL REPORT OF MULTISTATE RESEARCH PROJECT NE-1227
University of New Hampshire Agricultural Experiment Station
Department of Molecular, Cellular and Biomedical Sciences
2015 Annual Report

Project: NE-1227, Ovarian Influences on Reproductive Success in Ruminants.

Participants (FTE):

Individuals working on the project: Co-PIs – Paul C.W. Tsang (0.2), David H. Townson (0.2); Collaborators – Marsha A. Moses (0.1), Childrens' Hospital, Boston; John S. Davis (0.1), University of Nebraska-Omaha, Alan L. Johnson (0.1), Francisco J. Diaz (0.1), Olga M. Ocon-Grove (0.1), Pennsylvania State University; Catherine M. Combelles (0.1), Middlebury College; Graduate students – Christopher Cummings (0.5), Nicole Jaskiewicz (0.5), Joey Miseirvitch (0.5); Post-docs – Christine Coticchia (0.1), Children's Hospital, Boson.

Target Audience:

Target Audiences: Scientists working in similar areas, local and regional stakeholders, undergraduate and graduate students.

Efforts: Peer-reviewed abstracts and publications, conference presentations, formal classroom instruction, AES field-day presentations for livestock producers, laboratory instruction, curriculum development, experiential learning for students through research opportunities.

Products:

Peer-reviewed Manuscripts

Ott TL, Kamat MM, Vaudevan S, Townson DH, Pate JL (2014) Maternal immune responses to conceptus signals during early pregnancy in ruminants. Anim. Reprod. 11: 237-245.

Abstracts

Coticchia C, Miseirvitch J, Cummings C, Davis JS, Tsang PCW, Moses MA (2014) Regulation of cysteine rich 61-connective tissue growth factor-nephroblastoma overexpressed (CCN1) in granulosa cells. 47th Meeting of the Society for the Study of Reproduction in Grand Rapids, MI.

Townson DH, Diaz FJ, Ocon-Grove OM, Johnson AL (2014) A practical in vitro approach for the investigation of bovine granulosa cells from small follicles. 47th Meeting of the Society for the Study of Reproduction.

Jaskiewicz NJ, Parisi S, Hermawan C, Townson DH (2015) O-GlcNAcylation affects the tumorigenic potential of cervical cancer cells. American Society for Biochemistry and Molecular Biology Annual Meeting.

Fernandes N, Jaskiewicz NM, Chu F, Townson DH (2014) Determining the functional role of keratin filament in apoptosis via the PI3K/Akt signaling pathway using LTQ Orbitrap MS/MS analysis. Journal for the American Society for Mass Spectrometry Annual Conference, 25(1): 142.

Other Products:

1) Determined that KGN cells, a human granulosa carcinoma cell line, respond to supplemented estradiol by upregulating CCN1 expression. In addition, basic fibroblast growth factor, but not the platelet-derived growth factor homodimers (AA or BB), stimulated CCN1 expression.
2) Determined that bovine granulosa cells (bGCs) obtained from small follicles (3-5 mm) of ovarian pairs, and cultured under serum-free conditions, retain responsiveness to IGF-1 in terms of upregulation of CYP19A transcripts (qPCR) and conversion of testosterone to estradiol (ELISA).
3) Failed to demonstrate thus far that transduction of bGCs with constitutively-active MEK1 adenovirus increases MEK1 expression, ERK1/2 phosphorylation, and protects against cytokine-induced apoptosis in serum-free cultures.
4) Discovered that O-GlcNAcylation is important to the tumorigenic potential of SiHa cells (an immortal cervical cancer cell line) in that it enhances cell proliferation, augments cell viability, and amplifies cell migration/invasiveness. Preliminary proteomic analysis indicates that O-GlycNAclylation regulates elements of the anti-apoptotic, AKT pathway.

Accomplishments:

What was accomplished:

Objective 1: Identify inter-cellular interactions between somatic cells of the ovary, somatic cells and germ cells, or somatic cells and the embryo that promote follicular growth, oocyte maturation, early embryonic development, and establishment and maintenance of pregnancy. Toward this goal, the following was accomplished:

1) **A practical in vitro approach for the investigation of bovine granulosa cells from small follicles. DH Townson (UNH), FJ Diaz (Penn State), OM Ocon-Grove (Penn State), AL Johnson (Penn State)**
   The objectives were to 1) develop a serum-free culture method in which bovine granulosa cells (bGCs) derived from small follicles (3-5mm) of a single pair of ovaries are utilized, and 2) evaluate the hormonal responsiveness and steroidogenic capacity of the bGC model. In brief, ovarian pairs (slaughterhouse) were staged as early- to mid-estrous cycle according to morphologic criteria. All small, healthy antral follicles (3-5 mm; vascularized, clear fluid) of a given pair were aspirated to collect bGCs. The bGCs from each pair were seeded in T25 flasks with DMEM/F12 culture medium, 10% FBS and antibiotics, regardless of cell number/viability, and were incubated at 37C in 5% CO2 and 95% air for 3-4 days until 100% confluent. At confluency, the cells were trypsinized, counted and then seeded either in 96-well or 12-well plates, or in T25 flasks. The day after seeding, the cultures

were switched to serum-free DMEM/F12 medium containing insulin (10 or 100 ng/ml), transferrin (5.5 or 55 ng/ml) and sodium selenite (0.67 or 6.7 pg/ml) for the duration of the experiments. In summary, a serum-free culture method has been developed in which bGCs derived from small follicles (3-5 mm) of a single pair of ovaries can be maintained for up to six days. The bGCs proliferate in response to IGF1, and to a lesser extent FSH. The bGCs are sensitive to cytokine-induced apoptosis, but can be rescued by IGF1 treatment. CYP19A1 expression in the bGCs is upregulated by IGF1 and FSH treatment. FSHR and STAR expression in the bGCs is relatively low, but measurable after six days of culture. IGF1, and to a lesser extent FSH, stimulate estradiol secretion by the bGCs within 48 hr of treatment. Thus, the current, serum-free method of bGC culture provides for hormone-induced cell proliferation, cytokine-induced cell death, and measurement of the expression of hormone-responsive and steroidogenic genes in small follicles similar to that described in previously reported bGC culture models. Importantly, the current culture system utilizes bGCs originating from a single pair of ovaries rather than pooled ovaries. Thus, it preserves animal variation for experimental purposes, and has the potential to provide further insight about follicular dynamics during folliculogenesis in the cow for future work.

2) **Role of leukocyte adhesion molecules in recruitment of immune cells into the CL. L Steinberger (Penn State), JL Pate (Penn State), DH Townson (UNH)**

Hypothesis: Leukocyte adhesion molecules expressed on luteal endothelial cells vary with functional state of the CL, facilitating infiltration of immune cells only after the CL is fully functional. The objective of this experiment was to determine if prostaglandins alter adhesion molecule expression on endothelial cells of midcycle corpora lutea (days 12-15) compared to early corpora lutea (days 5-7) to allow for immune cell infiltration into the tissue. Luteal endothelial cells (LEC) were isolated from early and midcycle corpora lutea by size exclusion. Endothelial cells were cultured in RPMI + 20% fetal bovine serum (FBS) + gentamicin in 6 well plates for 72 hours to allow for cell attachment. After 72 hours, cells were washed and cultured for another 24-48 hours to allow for cell proliferation. When cells reached ~80% confluency, they were washed and cultured in RPMI + gentamicin + ITS overnight. Cells were treated with a physiological (10 ng/ml) or supraphysiological (1□M, which was 353-476 ng/ml, depending on the prostaglandin) concentration of each prostaglandin for 24 hours. Cells were collected and analyzed for concentration of vascular cell adhesion molecule 1 (VCAM1), selectin P (SELP) and chemokine (C-C motif) ligand 2 (CCL2) mRNA by qPCR. Additionally, LEC expression of prostaglandin receptor mRNA was determined by PCR. Tumor necrosis factor 1 (TNF; 10 ng/mL) and no treatment were used as controls.

PGE2 (PTEGR2, PTEGR4), PGF2□ (PTGFR) and PGI2 (PTGIR) receptor mRNA were expressed by LEC. Concentration of CCL2 mRNA was

reduced by both physiological and supraphysiological concentrations of PGF2□ in early LEC and by PGI2 in midcycle LEC. Both concentrations of PGE2 downregulated CCL2 in early and midcycle CL. VCAM1 was reduced by both concentrations of PGI2 in midcycle LEC only. Samples were analyzed for differences between low and high concentrations of prostaglandins. There was a significant difference between the two concentrations of PGE2 in the concentration of VCAM1 in midcycle LEC and in SELP concentration in early LEC. There was a tendency for a difference between concentrations of PGI2 in SELP concentration in early LEC. This is important because many studies have used 1 μM of prostaglandin, which is supraphysiological. In this study, effects on some mRNA were exaggerated by the supraphysiological concentration of PG.

TNF treatment upregulated the expression of CCL2 and VCAM1 by LEC isolated from early and midcycle luteal tissue and SELP by midcycle LEC. Previous reports have shown that CCL2 is upregulated by TNF (50-100 ng/mL) in CLENDOs of early pregnancy. However, these are the first data to demonstrate increased SELP and VCAM1 mRNA in response to TNF in LEC.

3) CCN1 Expression in a Human Ovarian Granulosa Cancer Cell Line
Christine Coticchia, Joey Miseirvitch, Christopher Cummings, John S. Davis, Marsha A. Moses and Paul C. W. Tsang.

Vascularization or angiogenesis of the ovulatory follicle is a hallmark event as it transitions to become the corpus luteum. Previously, we reported that the angiogenic inducer, Cysteine rich 61-Connective tissue growth factor-Nephroblastoma overexpressed 1 (CCN1; formerly known as CYR61), was highly expressed in the young, day 4 bovine corpus luteum. We hypothesized that CCN1 plays a key role in orchestrating the folliculo-luteal transition. To this end, we have shown that bovine granulosa and theca express CCN1, along with αV, αIIb, α6, β1, β3 and β5 integrin subunits. The heterodimeric integrins, e.g., αVβ3 and αVβ5, are receptors for CCN1.

Since angiogenesis is also a hallmark event in tumors, parallel studies were conducted using the KGN granulosa tumor cell line, which was established in 1994 from a postmenopausal woman who had recurring ovarian granulosa cell carcinoma. Similar to bovine granulosa and theca, KGN cells express CCN1 and its receptor integrin subunits, αV, α1, α3 α5, α6, β1, β3 and β5.

CCN1 is transcriptionally regulated by a wide variety of factors, which include, for example, serum growth factors, FGF2, platelet-derived growth factor, growth hormone, estrogens, cAMP and cytokines. In KGN cells, like bovine granulosa cells, CCN1 expression is rapidly induced within 1-2 hours by fetal bovine serum (FBS). However, little else is known about the regulation of CCN1 expression in granulosa cells. Thus, the objective of the present study was to determine the effects of estradiol and growth factors on

CCN1 expression in KGN cells. In Experiment 1, a preliminary study was conducted to determine the effects of Estradiol on CCN1 expression. KGN cells were grown to near confluency; the medium was removed; and the cells were rinse with modified Hank's balanced salt solution (HBSS) before the following treatments were applied: with fetal bovine serum (+FBS) or without FBS (–FBS), and in the absence or presence of estradiol (0.13, 0.013, 0.0013 or 0.00013 ug/mL). At the end of 2 hours, the medium was collected while the cells were extracted for RNA. Progesterone in the medium was determined by radioimmunoassay, while CCN1 expression was determined by real-time PCR. In Experiment 2, a preliminary study was conducted to determine the regulation of CCN1 expression by growth factors. KGN cells were grown to near confluency in 100 mm dishes before they were starved and stimulated for 2 hours with specific ligands, including FGF (5ng/mL), PDGF AA (10ng/mL) and PDGF BB (10ng/mL).

In Experiment 1, preliminary analysis revealed that estradiol may stimulate CCN1 expression by KGN cells. In Experiment 2, preliminary analysis revealed that PDGF AA and PDGF BB did not induce CCN1 expression beyond basal levels, while FGF2 treatment might upregulate CCN1 expression in KGN cells.

In summary, physiological concentration of estradiol and FGF2 may be regulators of CCN1 expression in KGN cells. Additional replicates are required to confirm these observations, and new experiments will be designed to determine the signaling molecules activated by FGF2 and other growth factors.

Opportunities for training and professional development:

Taught graduate and undergraduate students how to carry out the indicated experiments and analyze the resulting data; portions of these studies are part of a graduate student's dissertation project and several undergraduate research experience projects.

Students were trained by the PIs to develop cell cultures, extract protein/RNA, amplify RNA, and conduct immunoblotting, qPCR, and immunohistochemistry. The PI, CoPIs, and students developed poster and oral presentations and peer-reviewed publications based on the data collected during the study.

How results have been disseminated to target audience:

Graduate students and post-doc presented 2 abstracts at the Society for the Study of Reproduction (international meeting)

Undergraduate students presented abstracts at the annual UNH Undergraduate Research Conference

PIs presented data at the UNH Research Field Day (UNH) which was attended by members of the NH Agricultural Experiment Station External Advisory Board, local stakeholders, dairy extension agents, and UNH students and faculty.

Plans for Next Reporting Period:

A. Determine the regulation of CCN1 in bovine granulosa and theca by estradiol, FGF2 and other growth factors.
B. Determine the signaling pathway(s) associated with the regulation of CCN1 expression in KGN cells, and in bovine granulosa and theca, by FGFs and other growth factors.
C. Further refine/validate serum-free bovine granulosa cell culture model, and implement experiments to genetically alter the mitogen activated protein kinase (MAPK) pathway (Adenovirus overexpression of MEK1, siRNA knockdown of MEK1) in these cells.

Major changes: N/A

Support:
Hatch Regional/Multistate Funds, New Hampshire Agricultural Experiment Station.

University of Nebraska Experimental Station 2014-2015 Annual Report (NEB 26-206): NE1227 -Ovarian Influences on Reproductive Success in Ruminants

PARTICIPANTS (FTE)

Individuals working on the project: PI - Jennifer Wood (0.2), UNL; Co-PIs – Andrea S. Cupp (0.1), UNL; John S. Davis (0.1), UNMC; Bob Cushman (0.1) US MARC; Technicians at UNL – Scott Kurz (0.25) and Jeff Bergman (0.1); Graduate students at UNL – Fang Xie (0.25), Mohamed Ayoub (0.25), William Pohlmeier (0.25), Renata Spuri-Gomes (0.25), and Renee McFee (0.1); Post-docs at UNL – Adam Summers (0.3) and Sarah Romereim (0.5)

TARGET AUDIENCE

Target Audiences: Scientists working in complementary areas, producers, graduate students, technicians, undergraduate students, and veterinary medicine students.

Efforts: conference and meeting presentations, peer-reviewed manuscripts, formal classroom instruction, laboratory instruction, development of curriculum and case study scenarios, experiential learning opportunities.

PRODUCTS

Peer-reviewed Manuscripts

1. Pohlmeier WE, Xie F, Kurz SG, Lu N, and **Wood JR** (2014) Progressive obesity alters the steroidogenic response to ovulatory stimulation and increases the abundance of RNAs stored in the ovulated oocyte, *Mol Reprod Dev* 81(8): 735-747, PMID:24824196
2. Baier SR, Nguyen C, Xie F, **Wood JR**, and Zempleni J (2014) MicroRNAs are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow milk and affect gene expression in peripheral blood mononuclear cells, HEK-293 kidney cell cultures, and mouse livers *J Nutr* 144(10): 1495-1500, PMCID: PMC4162473
3. Summers AF, Pohlmeier WE, Brauer VM, Sargent KM, Kurz SG, McFee RM, Cushman RA, Cupp AS, and **Wood JR** (2014). Altered theca gene expression and ovarian follicular development in cows with follicular fluid androgen excess *PLoS ONE* 9(10) e110683, PMCID: PMC4199720
4. Cushman RA, Tait Jr. RG, McNeel AK, Forbes ED, Amundson OL, Lents CA, Lindholm-Perry AK, Perry GA, **Wood JR**, Cupp AS, Smith TPL, Freetly HC, and Bennett GL (2015) A polymorphism in myostatin influences puberty but not fertility in beef heifers, whereas μ-calpain affects first calf birth weight. *J Anim Sci* 93(1)117-126, PMID: 255568362

Abstracts

1. Xie F, Timme K, Rifer JL, and **Wood JR** (2014) Diet-induced obesity increases of Pou5f1 and Dppa3 in growing and mature oocytes are due to increased STAT3 signaling. *11th Annual Gilbert Greenwald Symposium on Reproduction*, Kansas City, KS
2. Romereim S, Summers AF, Pohlmeier WE, McFee R, Spuri-Gomes R, Kurz SG, Cushman RA, Davis JS, **Wood JR**, and Cupp AS (2014) Differential regulation of cell cycle and immune response networks in bovine granulosa cells with excess intrafollicular

androstenedione. *11th Annual Gilbert Greenwald Symposium on Reproduction*, Kansas City, KS

3. Spuri-Gomes R, Tenley SC, Kurz SG, **Wood JR**, and Cupp AS (2015) Cows with intrafollicular androgen excess have lower sex hormone binding globulin and appear to be chronic or sporadic anovulatory. *Society for the Study of Reproduction 48th Annual Meeting*, San Juan, Puerto Rico
4. Romereim SM, Summers AF, Pohlmeier WE, McFee RM, Spuri-Gomes R, Kurz SG, McNeel AK, Cushman RA, Davis JS, **Wood JR**, and Cupp AS (2015) Granulosa cell cycle regulation and steroidogenesis in a high androstenedione follicular microenvironment. *Society for the Study of Reproduction 48th Annual Meeting*, San Juan, Puerto Rico
5. Ayoub M, Manamage M, Vinton R, Cushman RA, McNeel AK, Freetly HC, **Wood JR**, and Cupp AS (2015) Vascular endothelial growth factor A 165 (VEGFA165), angiogenic isoform, promotes while VEGFA165b antagonizes VEGFA165 stimulated follicular progression in bovine ovarian cortical pieces cultured from pre-pubertal heifers. *Society for the Study of Reproduction 48th Annual Meeting*, San Juan, Puerto Rico
6. Xie F, Anderson CL, Timme K, Fernando SC, and **Wood JR** (2015) Increased *Firmicutes* in the cecum of obese female mice is correlated with increased Pou5f1 and Dppa3 mRNAs in growing oocytes which may be mediated by increased Toll-like receptor 4 signaling in the ovary. *Society for the Study of Reproduction 48th Annual Meeting*, San Juan, Puerto Rico

Invited Talks

1. **Wood JR** (2014) Departmental Seminar, Animal Science, Iowa State University; Ames, IA; Influence of Ovarian Environment on Oocyte Growth and Maturation: Lessons Learned from Cows and Mice

OTHER PRODUCTS

1) Developed a fluorescent in situ hybridization protocol to detect and quantitate the number of specific mRNAs in individual growing and mature oocytes
2) Established a protocol in our laboratory for chromatin immunoprecipitation of whole ovary and purified ovarian somatic cells. This assay measures association of proteins with promoter regions of genes
3) Collected transcriptome data from granulosa, theca, small luteal, and large luteal cells which were obtained from High A4 and Low A4 follicles
4) Developed protocols in our laboratory to stain lipid droplets, organelles, and transzonal projections in the bovine cumulus oocyte complex

ACCOMPLISHMENTS

Impact: Developing replacement heifers and establishing a pregnancy which results in the birth of a live calf are the two major costs to beef and dairy producers. Indeed, pregnancy loss costs producers 1.2-1.4 billion dollars annually in the US. Infertility also affects approximately 12% of US women (i.e. 7.3 million) with 22% of these cases attributed to ovarian dysfunction (CDC). These women are disproportionately obese and/or have metabolic dysfunction including the androgen excess disorder Polycystic Ovary Syndrome suggesting a link between metabolic stress and ovarian dysfunction. Thus, the **long term goal** of this project is to determine how

metabolic stress (1) alters the environment surrounding the growing oocyte; (2) impacts inter-cellular interactions between somatic cells of the ovary (i.e. theca and granulosa) as well as somatic cells and the oocyte (i.e. granulosa and oocyte); and (3) impairs intracellular signaling pathways and gene expression regulatory mechanisms which collectively determine the effectiveness of oocyte growth and maturation. Our current studies have established novel animal models of metabolic stress which have been used to generate changes in knowledge regarding the steroids which are produced by somatic cells of the ovary and changes in oocyte gene expression associated with reduced oocyte quality. These discoveries have uncovered potential therapeutic targets that can be manipulated in order to improve ovarian function and successful establishment of pregnancy in cattle and women.

What was accomplished

OBJECTIVE 1

Increased Firmicutes in the Cecum of Obese Female Mice is correlated with Increased *Pou5f1* and *Dppa3* mRNAs in Growing Oocytes which may be Mediated by increased Toll-like Receptor 4 signaling in the Ovary

Fang Xie, Christopher L. Anderson, Kelsey Timme, Samodha C. Fernando, and Jennifer R. Wood

Our previous studies showed that embryo development is reduced and oocyte mRNA abundance is increased in a mouse model of obesity. Specifically, *Dppa3* and *Pou5f1* were increased 2-fold ($P<0.02$) in growing oocytes and 6- ($P<0.024$) and 12-fold ($P<0.033$), respectively, in ovulated oocytes collected from obese compared to lean mice. Chromatin immunoprecipitation also indicated obesity-dependent increases in *Dppa3* and *Pou5f1* promoter activity in growing oocytes. However, obesity-dependent mechanisms of increased *Dppa3* and *Pou5f1* transcription have not been defined. Recently the relationship between gut microbial communities and obesity has been demonstrated. Indeed, the ratio of Bacteriodetes to Firmicutes, which represent the major phyla of gut microbes, is typically decreased in obese individuals and is associated with the development of chronic inflammation and increased lipid deposition. Despite the explosion of studies documenting the impact of the gut microbiota on the function of metabolically important tissues, studies linking the community structure of gut microbes and markers of ovarian function are lacking. Thus, the objective of the current study was to determine if obesity-dependent increases in oocyte mRNA abundances is correlated to differences in the gut microbiome. For this study, C57BL/6J (B6) or DBA2J (D2) mice were maintained on normal rodent chow (ND; 13% kcal fat) or a high fat diet containing either 45% (45HFD) or 60% (60HFD) of kcal from fat for 12 weeks. At 17 weeks of age, ovulation was induced using exogenous gonadotropins. Ovulated oocytes, whole ovary, adipose tissue, and cecum contents, were collected from each animal 16h post-hCG. There were no differences in ovulation rate or ovarian weight between experimental groups and all ovaries were only populated with corpora lutea and similar numbers of growing, transcriptionally active oocytes. As expected, abdominal adipose tissue weight was increased in B6 and D2 mice fed a 45HFD or 60 HFD indicative of an obese phenotype. The microbial community structure of each mouse was evaluated by amplifying and sequencing the V3 region of the 16S rRNA gene in cecum contents using an Ion Torrent PGM to a depth of ~13,000 reads per sample. The resulting quality passed reads were clustered using UPARSE and were further analyzed using Quantitative Insights Into Microbial Ecology (QIIME). Multivariate correlation analysis was performed using Multivariate Association with Linear Models (MaAsLin). The MaAsLin showed that ovarian *Dppa3* mRNA

was positively correlated with OTU1418 (P<0.0016) while ovarian *Pou5f1* mRNA was positively correlated with, OTU328 (P<0.0009) and OTU1433 (P<0.0032). These 3 species of bacteria were classified as belonging to the phyla Firmicutes and family Lachnospiraceae at 80 % confidence based on the RDP "classifier" algorithm. To determine if the diet induced obesity also altered inflammatory markers in the ovary, qPCR analysis of *Tnfa, Tlr4, Il6*, and *Il1b* was carried out. *Tnfa* and *Tlr4* mRNA was increased 2-fold (*P*<0.05) in whole ovary of 60HFD B6 females; however, no difference in *Il6* or *Il1b* transcripts were detected. Together, these data suggest a potentially novel mechanism by which gut microbes influence the expression of ovarian genes and thereby contribute to the quality of oocyte development. Furthermore, manipulation of the gut microbiome may represent a therapeutic strategy to improve pre-conception care in overweight and obese women.

Cows with Intrafollicular Androgen Excess have Lower Sex Hormone Binding Globulin and appear to be Chronic or Sporadic Anovulatory

Renata Spuri-Gomes[1], Sarah C Tenley[1], Scott G. Kurz[1], Jennifer R.Wood[1], Andrea S. Cupp[1]

We have identified a population of cows with excess intrafollicular concentrations of androstenedione (A4; >30 fold) compared to their control counterparts with no similar fold increase in estrogen levels. Furthermore, these cows have reduced calving rates (17%; P < 0.07) suggestive of female subfertility. Therefore, our objective with the current study was to investigate endocrine and follicular profiles in cows with Low intrafollicular A4, (Low A4; < 20 ng/ml; control) and High A4 (>20ng/ml) during a reproductive cycle. After classification of cows as High A4 (n=6) and Low A4 (n=5), their estrous cycles were synchronized with an injection of prostaglandin (25mg) to lyse the CL and induce ovulation and the start of a new reproductive cycle. Each day for a period of 28 days ovarian follicular dynamics were evaluated and recorded by transrectal ultrasound examinations. Jugular blood samples were also taken daily to determine plasma progesterone and androstenedione concentrations to better characterize their endocrine profiles. No difference was observed in the length of the estrous cycle, nor on the size of the dominant ovulatory follicle. Interestingly, we found that some animals within the High A4classification displayed estrus but did not ovulate, while others ovulated but did not display estrus. One cow had undetectable progesterone (<1 ng/ml) throughout the study, which would be indicative of a chronic anovulatory P4 profile and this cow also had a persistent follicle that remained dominant on the ovary for approximately 9 days. In order to better understand these phenotypes, serum hormone analyses for progesterone (P4; daily during the estrous cycle), sex hormone binding globulin (SHBG; days 7 and 15 of the estrous cycle) and A4 (days 7 and 15 of the estrous cycle) were conducted. The area under the curve (AUC) for P4 throughout the estrous cycle was not different between the two groups but the pattern of progesterone secretion in High A4 cows was similar to women with sporadic anovulation with a 40% decrease in peak value of progesterone (5.83 ng/ml) vs Low A4 (8.18 ng/ml). In addition, P4 values in Low A4 cows dropped below 1.5 ng/ml from day 22 until day 27 while High A4 cows remained at >3.0 ng/ml. In women with androgen excess SHBG has been demonstrated to be decreased; therefore, we analyzed it on days 7 and 15 of the estrous cycle. In both groups of cows SHBG increased from day 7 to 15 (Low A4 cows; p=0.0074 and High A4 cows; p=0.0377), but at day 15; the High A4 animals have lower levels of SHBG (p= 0.0076) compared to Low A4 cows. Concentrations of total A4 were not different at either day 7 or 15 between A4 groups; however, the values of A4 increased significantly in High A4 cows at day 15 when compared to day 7 with no difference in A4 between Day 7 and 15 in the Low A4 cows. Thus with decreased SHBG

more free A4 would be available in the High A4 cows to act. Taken together our data indicate that the High A4 cows have reduced peak values of P4 during the estrous cycle with progesterone concentrations remaining elevated over a longer period of time in the High A4 cows. This sustained concentration of P4, and elevated free A4 due to reduced SHBG may be contributing to development of follicles which may not respond as well to ovulation. Furthermore, SHBG at mid-cycle may be a potential predictive marker of some types of anovulatory disorders (ie., sporadic or chronic anovulation) which are indicators of female infertility.

Granulosa Cell Cycle Regulation and Steroidogenesis in a High Androstenedione Follicular Microenvironment

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Anovulatory infertility (either chronic or sporadic anovulation) affects up to 40% of infertile women. In fact, sporadic anovulation in humans may often go undetected since recent literature has reported that 8-13% of normally menstruating women (250 total, two reproductive cycles) exhibit sporadic anovulation. To gain a greater understanding of anovulation, our lab has identified a naturally-occurring bovine model system which includes a subpopulation of cows with a 17% reduction in calving rate ($P < 0.07$) as well as endocrine profiles and ultrasound-tracked follicular development profiles that resemble those of women with chronic or sporadic anovulation. These cows exhibit excess androstenedione (A4) accumulation in their follicular fluid (10%; >30 fold higher) and lack an increase in estrogen production of similar magnitude (only 2 to 4 fold higher). Our objective was to gain a mechanistic understanding of the causes and consequences of this high-androgen follicular microenvironment.

In pursuit of this objective, our lab performed microarray analysis using Affymetrix Bovine GeneChip 1.0 ST on the granulosa cells from control Low A4 (n=4, follicular A4 <20 ng/mL) and High A4 (n=5, follicular A4 > 40 ng/mL) cows. Overall, based on ANOVA array statistical testing ($P<0.005$ and FDR<0.05) there were 1229 genes upregulated and 255 downregulated in granulosa cells from High A4 cows compared to Low A4 cows. Using Ingenuity Pathway Analysis, we found that granulosa cells from the High A4 population exhibit a strong inhibition of the cell cycle, especially G1/S checkpoint proteins (e.g. cyclins and cyclin-dependent kinases), regulators of chromosome alignment and segregation (e.g. kinesins and related molecules), and other cell cycle regulators. Validation of the microarray results with qRT-PCR has confirmed the differential regulation of several genes. For example, CCNA2 (cyclin A2) regulates cell cycle checkpoint progression and was downregulated in High A4 granulosa, suggesting that those cells may be undergoing cell cycle arrest. An additional gene, ECT2, is required for signal transduction pathways that control cytokinesis and is expressed in a cell cycle-dependent manner (with expression during DNA synthesis through cytokinesis). ECT2 is downregulated in High A4 granulosa, which likely suggests that fewer cells in the sampled population are in S Phase, G2, or mitosis and/or that cytokinesis may be impaired. Moderate increases in genes associated with lipid intake (membrane transporters) and lipid metabolite breakdown (UGT family) in High A4 cows suggest increased steroidogenesis in granulosa cells from High A4 vs Low A4 cows. This cow population with excess intrafollicular androgen and sporadic anovulation is an excellent model to study mechanisms involved in female anovulatory infertility and ultimately develop therapeutics and procedures to enhance reproductive success in women. Furthermore,

this bovine population can also be used to develop infertility markers in agriculturally relevant species which may be used in selection programs to ensure better reproductive success within the livestock industry.

Vascular Endothelial Growth Factor A 165 (VEGFA165), Angiogenic Isoform, promotes while VEGFA165b antagonizes VEGFA165 stimulated follicular progression in bovine ovarian cortical pieces cultured from pre-pubertal heifers

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Previously we have demonstrated in our lab that the VEGFA165 angiogenic isoform stimulates follicle progression in perinatal rat ovaries while inhibitors to VEGFA signal transduction pathways arrest follicular development. Thus, in the current study our objective was to determine if the anti-angiogenic VEGFA165b antagonizes the stimulatory effects of VEGFA165 angiogenic isoform in ovarian cortex from pre-pubertal heifers and thereby affect the ability of follicles to progress.

Six pre-pubertal heifers were ovariectomized at 8-9 months and ovarian cortical strips were collected. RNA was collected from Day 0 cortical pieces before culture to measure the expression of angiogenic genes using Quantitative PCR (QPCR). There were no differences in any of genes: KDR, PECAM, VE-cadherin and NRP-1 suggesting no differences in vasculature prior to ovarian cortex culture. The cortical strips were cultured for 7 days in Waymouth's MB 752/1 medium supplemented with antibiotics, ITS and BSA. This medium was then either supplemented with 50 ng/ml of angiogenic VEGFA165 (rhVEGF165; R&D Systems) alone, antiangiogenic VEGFA165b (rhVEGF165b; R&D Systems) alone, or a combination of VEGFA165 and VEGFA 165b; or control (PBS). After the 7 days of culture, the cortical strips were fixed and embedded in paraffin and 4-5 serial sections of 5µm thickness were taken from each block. Slides of cortex sections were stained with hematoxylin and eosin (H&E) for follicle staging. Follicle stages were conducted using the following rubric: stage 0 (primordial follicle): An oocyte surrounded by a single layer of squamous pregranulosa cells, stage 1(transitional follicle): An oocyte surrounded by mostly squamous pregranulosa cells with some cuboidal cells; stage 2 (primary follicle): An oocyte surrounded by 1-1.5 layers of cuboidal granulosa cells; stage 3 (secondary follicle): An oocyte surrounded by 2 or more layers of cuboidal granulosa cells; stage 4 (tertiary follicle): An oocyte surrounded by 2 or more layers of granulosa cells that contains a distinct antrum. Three different technicians counted 3 slides for each treatment and results were averaged per treatment. Neither VEGFA isoform treatment had an effect on numbers of primordial follicles (stage 0). However, there was an increase in the number of primary follicles (stage 2) in the VEGFA165 (0.55±0.02; $P < 0.0001$) treatment vs any other treatment: VEGFA165b (0.25±0.02), Combination (0.24 ±0.02), and PBS (0.20±0.02). Furthermore, there was also an increase in the number of secondary follicles (stage 3) that were in the bovine cortex treated with VEGFA165 (0.112±0.001; $P < 0.0001$) vs the other treatments: VEGFA165b (0.019±0.001), Combination (0.010 ±0.001), and PBS (0.037±0.001). Thus, we can conclude from these results that VEGFA isoform, VEGFA165, can stimulate follicle progression in bovine cortex which supports previous data in our lab. Interestingly, VEGFA165b inhibited VEGFA165-dependent stimulation of follicular progression to stages 2 and 3 suggesting that VEGFA165b can antagonize the actions of angiogenic isoform VEGFA165. Therefore, these bovine cortex cultures are an excellent model to understand the antagonistic mechanisms of

VEGFA165b, the antiangiogenic isoform, on the ability of VEGFA165 to stimulate follicle progression. It is possible that VEGFA165b contributes to the block in follicle development in the ovary to maintain the ovarian reserve and guard against premature ovarian failure.

OBJECTIVE 2

No data was collected for this objective during the current reporting period

Opportunities for training and professional development

Training: Students were trained by the PI, technicians, and/or collaborators to dissect, fix, embed, and section whole ovaries from lean and obese mice. Collection of oocytes and zygotes as well as culture of zygotes to the 2-cell stage was also taught. Students learned how to collect RNA and carry out qPCR, collect protein extracts and carry out Western blot analyses, and collect chromatin crosslinked with proteins to carry out chromatin immunoprecipitation assays. Graduate and undergraduate students were taught how to analyze the resulting data from these experiments. A portion of these studies were part of a 2 graduate student theses, 2 graduate student dissertations, and 2 undergraduate research experience projects.

Professional development: Graduate student and post-doc attended and presented data at a regional (Greenwald Symposium) symposium while PI, post-doc and graduate student attended and presented at the Society for the Study of Reproduction annual meeting. Trainee mentoring sessions were also attended at the SSR meeting by the post-doc and graduate student. The PI additionally served as chair of the Awards Committee at this meeting. Undergraduates attended and reported data at the UNL Research Fair. PI and graduate students participated in weekly and monthly seminar series which included external speakers and student presentations.

How results have been disseminated to target audience

Manuscripts were published in peer-reviewed journals (4). Graduate student and post-doc presented data at the Society for the Study of Reproduction (international meeting) during platform sessions. Graduate student and post-doc presented data at the regional Greenwald Symposium. PI presented data to the UNL and Iowa State Animal Science Department including faculty, staff, and graduate students/postdocs. PI was a guest lecturer at a local high school (Milford HS, Milford, NE) and hosted a student for a job shadowing experience. PI, co-PI, and students participated in additional events to recruit students to STEM fields including agricultural sciences.

Plan for next reporting period

Objective 1

1. Define specific microbial species and metabolic factors produced by the microbes that influence oocyte gene expression and post-transcriptional regulation of mRNA stability during oocyte growth and maturation as well as immediately after ovulation and during the maternal zygotic transition.
2. Determine how excess maternal RNAs (e.g. Pou5f1, Dppa3) impact activation of the embryonic genome, development to the blastocyst stage, and the processes of gastrulation and neurulation.

3. Identify differences in intracellular signaling and the association of transcriptional activators vs. repressors with the promoter region of steroidogenic enzymes in theca cells collected from High v. Low A4 cows in order to identify mechanisms of androgen excess development.

Objective 2

1. Establish co-cultures of theca and granulosa cells from high and low A4 cows to determine how inter-cellular communication between these somatic cells coordinately regulates androgen and estrogen synthesis in the follicle
2. Determine the effect of female obesity on the temporal dynamics of transzonal projections which are crucial for intercellular communication between cumulus granulosa cells and the oocyte during growth and maturation.

University of Wisconsin-Madison Experimental Station 2014 Annual Report (NE1227): Ovarian Influences on Reproductive Success in Ruminants

Participants (FTE)

Individuals working on the project: PI - Milo Wiltbank (0.2), Paul Fricke (0.0) University of Wisconsin-Madison; Graduate students at University of Wisconsin-Madison –Giovanni Baez (0.5) and Alvaro Garcia-Guerra (0.5)

Target Audience

Target Audiences: Veterinarians and producers working in the dairy industry. Scientists working in reproductive physiology.

Efforts: Practical research on commercial dairy herds, fundamental research into understanding the ovary, peer-reviewed manuscripts in scientific journals, presentations at scientific conferences and industry meetings, Formal classroom and laboratory instruction

Products:

Peer-reviewed journal articles published in 2014 reporting research from this project

1. Bender RW, Hackbart KS, Dresch AR, Carvalho PD, Vieira LM, Crump PM, Guenther JN, Fricke PM, Shaver RD, Combs DK, **Wiltbank MC**. 2014. Effects of acute feed restriction combined with targeted use of increasing luteinizing hormone content of follicle-stimulating hormone preparations on ovarian superstimulation, fertilization, and embryo quality in lactating dairy cows. *Journal of Dairy Science* 97:764-778.
2. Ferraretto LF, Gencoglu H, Hackbart KS, Nascimento AB, Dalla Costa F, Bender RW, Guenther JN, Shaver RD, **Wiltbank MC**. 2014. Effect of feed restriction on reproductive and metabolic hormones in dairy cows. *Journal of Dairy Science* 97:754-763.
3. Nascimento AB, Souza AH, Keskin A, Sartori R, **Wiltbank MC**. 2014. Lack of complete regression of the Day 5 corpus luteum after one or two doses of PGF2α in nonlactating Holstein cows. *Theriogenology* 81:389-395.
4. Pereira MHC, Rodriguez ADP, De Carvalho RJ, **Wiltbank MC**, Vasconcelos JLM. 2014. Increasing length of an estradiol and progesterone timed artificial insemination protocol decreases pregnancy losses in lactating dairy cows. *Journal of Dairy Science* 97:1-11.
5. **Wiltbank, MC** and Pursley JR. 2014. The cow as an induced ovulator: Timed AI after synchronization of ovulation. *Theriogenology* 81:170-185.
6. Carvalho PD, Souza AH, Amundson MC, Hackbart KS, Fuenzalida MJ, Herlihy MM, Ayres H, Dresch AR, Vieira LM, Guenther JG, Grummer RR, Fricke PM, Shaver RD, **Wiltbank MC**., 2014. Relationships between fertility and postpartum changes in body condition and body weight in lactating dairy cows. *Journal of Dairy Science* 97:1-18.
7. **Wiltbank MC**, Souza AH, Carvahlo PA, Cunha AP, Giordano JO, Fricke PM, Baez GM, Diskin MG, 2014. Physiological and practical effects of progesterone on reproduction in dairy cattle. *Animal* 8:70-81.
8. **Wiltbank, M. C.**, A. Garcia-Guerra, P. D. Carvalho, K. S. Hackbart, R. W. Bender, A. H. Souza, M. Z. Toledo, G. M. Baez, R. Surjus, and R. Sartori. 2014. Effects of energy and protein nutrition in the dam on embryonic development. *Animal Reproduction* 11:168-182.

9. Carvalho, PD, Guenther JN, Fuenzalida MJ, Amundson MC, **Wiltbank MC**, FrickePM, 2014. Presynchronization using a modified Ovsynch protocol or a single gonadotropin-releasing hormone injection 7 d before an Ovsynch-56 protocol for submission of lactating dairy cows to first timed artificial insemination. *Journal of Dairy Science* 97:6305-6315.

Abstracts presented at scientific meetings during 2014 reporting research from this project

American Dairy Science Association - ADSA

1. P. D. Carvalho, N. E. Lobos, M. Z. Toledo, E. Trevisol, V. G. Santos, R. V. Barletta, G. M. Baez, A. Garcia-Guerra, J. N. Guenther, A. H. Souza, D. Luchini, P. M. Fricke, R. D. Shaver, and M. C. Wiltbank. 2014. Changes in plasma methionine concentrations after administration of two different doses of rumen protected methionine. Abstract # 1539.

2. N. E. Lobos, G. A. Broderick, P. D. Carvalho, D. N. Luchini, R. D. Shaver, A. H. Souza, and M. C. Wiltbank. 2014. Amino acid analysis in dairy cow plasma by chloroformate derivatization and gas chromatography. Abstract # 1546.

3. P. D. Carvalho, A. H. Sousa, M. C. Amundson, K. S. Hackbart, A. R. Dresch, L. M. Vieira, J. N. Guenther, R. R. Grummer, R. D. Shaver, P. M. Fricke, and M. C. Wiltbank. 2014. Relationship between fertility and postpartum changes in body condition and body weight in lactating dairy cows. Abstract # 337.

4. A. H. Souza, P. D. Carvalho, C. M. Drake, R. D. Shaver, and M. C. Wiltbank. 2014. Ration composition in Wisconsin dairy herds: Factors affecting fertility. Abstract # 1851. ADSA

5. P. D. Carvalho, M. J. Fuenzalida, A. Ricci, M. Luchterhand, J. M. Mulcahy, R. V. Barletta, G. M. Baez, V. G. Santos, M. C. Amundson, J. N. Guenther, A. H. Sousa, M. C. Wiltbank, and P. M. Fricke. 2014. Modifications to Ovsynch improve fertility during resynchronization: Evaluation of presynchronization with GnRH 6 days before Ovsynch and addition of a second PGF treatment. Abstract # 524.

Society for the Study of Reproduction - SSR

6. M. C. Wiltbank, P. J. Hansen, J. E. P. Santos. 2014. How knowledge of bovine reproductive physiology and development of reproductive technologies will help to feed a growing world population. Abstract # 72.

7. G. M. Baez, R. V. Barletta, A. Ricci, E. Trevisol, A. Garcia-Guerra, P. D. Carvalho, J. N. Guenther, B. O. Cardoso, M. Z. Toledo, M. C. Wiltbank. 2014. Treatment with GnRH 5d after Breeding Preferentially Produces an Accessory CL Contralateral to the Pregnancy and this CL Generally Regresses Prior to Day 75 of Pregnancy in Lactating Dairy Cows. Abstract # 206.

Brazilian Embryo Transfer Society – SBTE

8. G .M. Baez, R. V. Barletta, B. O. Cardoso, E. Trevisol, M. C. Wiltbank. 2014. Uterine size matters for fertility in lactating dairy cows. Abstract # A079. Selected as Second-Place Abstract in Practical Reproduction.

9. A. H. Souza, C. D. Narciso, E. O. S. Batista, P, D. Carvalho, M. C. Wiltbank. 2014. Effect of uterine environment on embryo production and fertility in cows. Abstract # 159.

10. M. C. Wiltbank, A. Garcia-Guerra, P. D. Carvalho, K. S. Hackbart, R. W. Bender, A. H. Souza, M. Z. Toledo, G. M. Baez, R. S. Surjus, R. Sartori. 2014. Effects of energy and protein nutrition in the dam on embryonic development. Abstract # 168.

11. M. C. Wiltbank, G. M. Baez, J. L. M. Vasconcelos, M. Pereira, A. H. Souza, R. Sartori, J. R. Pursley. 2014. The physiology and impact on fertility of the period of proestrus in lactating dairy cows. Abstract # 225.

Other Products

1) Alvaro Garcia-Guerra projects:
    a. Multiple ovulation genotype in cattle:

A high fecundity bovine genotype has been discovered. Carriers of this allele have multiple ovulations (MO) and half-sibling, non-carriers generally have single ovulations (SO). Based on fine-mapping to a region of chromosome 10 containing SMAD genes, we hypothesized that carriers of the MO genotype would have reduced follicle growth rate and earlier follicle differentiation than SO animals. Two experiments were performed to characterize the profiles of circulating hormones and follicular dynamics for MO (n=9) compared to SO (n=5) cattle. In experiment 1, a synchronized follicular wave was induced by follicular ablation with follicle growth in a controlled progesterone (P4) environment (no CL, one intravaginal P4 implant for 5 d, P4 removal to allow ovulation). In experiment 2, a complete interovulatory interval was evaluated. Circulating FSH, P4, and estradiol (E2) were evaluated by RIA and size of follicles and CL were determined by ultrasound every 12 h (Expt 1; ablation to ovulation) or every 24 h (Expt 2; complete interovulatory interval). In experiment 1, number of ovulations was greater (P=0.0003) for MO (4.0±0.4) than SO (1.6±0.2) as expected. Antral follicle count at wave emergence was not different (21.6±2.3 vs 22.8±2.2, P=0.705; MO vs. SO). Consistent with previous experiments in high fecundity ovine genotypes, mean ovulatory follicle size was greater (P=0.0004) for SO (15.7±0.8 mm) than MO (9.5±0.6) animals. Of particular interest, mean follicle growth rate was greater (P=0.0021) for SO (1.47±0.11 mm/day) than MO (0.97±0.07 mm/day) cows. Peak FSH concentrations were similar (0.66±0.04 vs 0.70±0.06 ng/ml, P=0.65, for MO and SO) with declining but similar FSH during the next 2 d for MO and SO. However, nadir FSH concentrations (72 h after final follicle aspiration until CIDR removal) were greater (P=0.023) for MO (0.25±0.02 ng/ml) than SO (0.17±0.02 ng/ml) cows. Mean E2 concentrations during the first 48 h after wave emergence were greater (P=0.03) in MO than SO but were not different after this time (P=0.197). In experiment 2, length of estrous cycle was not different between genotypes (22.1±0.9 vs 24.0±1.2 d, P=0.258, MO vs. SO). Number of ovulations for first (4.0±0.5 vs 1.2±0.2, P=0.002) and second (3.8±0.4 vs 1.2±0.2, P=0.004) ovulatory events were greater for MO than SO animals (Table 1). Following ovulation, there was no difference between genotypes (MO vs. SO) in luteal volume (day 10 = 4505.7±524 vs 6458±1380 mm^3; P=0.243), circulating P4 concentrations during the first 14 d of the estrous cycle (P=0.751), or maximal serum P4 (8.12±0.68 vs 8.73±1.2 ng/ml, P=0.676). As expected, volume of the largest ovulatory follicle and the largest CL was greater (P=0.004) for SO than MO animals, however circulating P4 during luteolysis (P=0.976) and peak circulating E2 (P=0.301) did not differ between genotypes. Interval from onset of estrus until ovulation also did not differ between genotypes (30.6±2.1 vs 26.4±2.4 h, P=0.214; MO vs. SO). During the first follicular wave, peak FSH was similar (P=0.285), although FSH was greater in MO than SO during the FSH decline (P=0.022) and FSH Nadir (P=0.009). Thus, MO cows have reduced rate of follicle growth in spite of similar or sometimes greater FSH concentrations, consistent with reduced rate of FSH-induced granulosa cell proliferation in individual follicles. Increased E2 from smaller follicles is consistent with differentiation of granulosa cells to a dominant phenotype at a smaller follicle size in MO than SO genotype.

Table 1. Comparison of various measures of follicular dynamics in carriers and non-carriers of the Trio genotype.

Genotype	Carrier	Non-carrier	P-value
Sample size	**9**	**5**	
N of ovulations	**4.0 ± 0.41**	**1.6 ± 0.2**	0.00003
Mean size ovulatory follicle (mm)	**9.5 ± 0.7**	**15.7 ± 0.8**	0.0002
Mean size largest follicle (mm)	**10.4 ± 0.7**	**16.4 ± 0.6**	0.0002
Mean size smallest follicle (mm)	**8.5 ± 0.7**	**15.0 ± 1.2**	0.002
Interval estrus-ovulation (h)	**30.6 ± 2.1**	**26.4 ± 2.4**	0.212
Mean individual volume ovulatory follicle (mm^3)	**503 ± 111**	**2111 ± 306**	0.004
Mean total volume ovulatory follicle (mm^3)	**1760 ± 233**	**3158 ± 439**	0.03
Mean growth rate largest follicle (mm/day)	**0.96 ± 0.07**	**1.47 ± 0.11**	0.006
Mean growth rate ovulatory follicles (mm/day)	**0.87 ± 0.04**	**1.39 ± 0.08**	0.0002
Antral follicle count	**21.6 ± 2.3**	**22.8 ± 2.2**	0.903

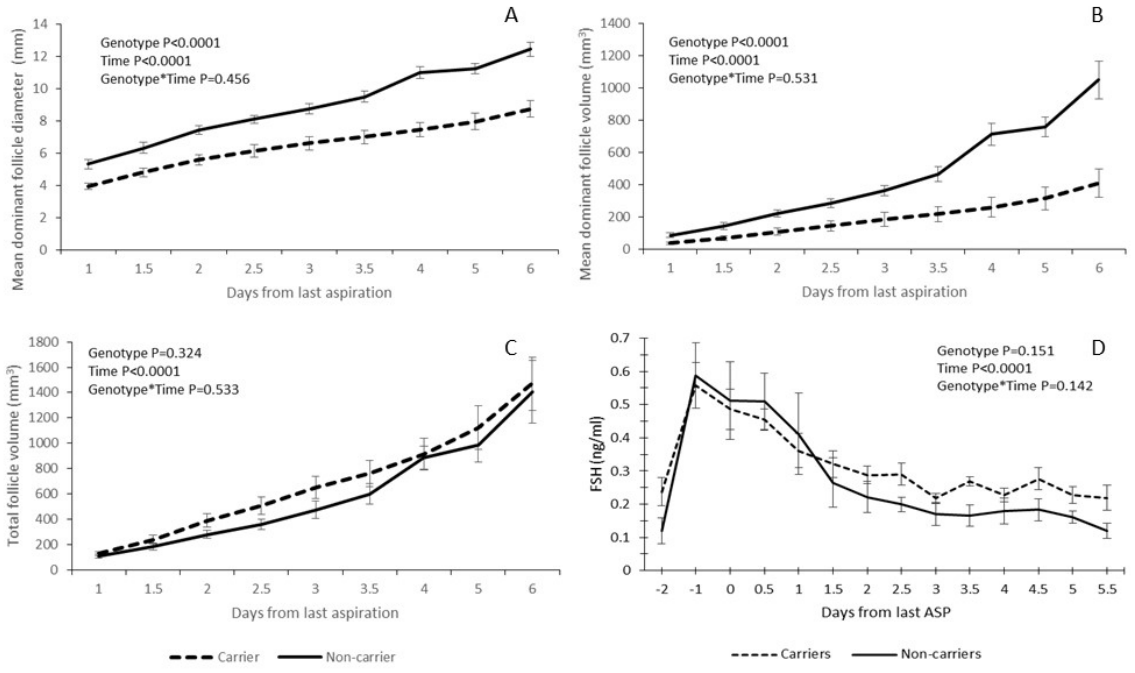

Figure 1.Follicle growth patterns in carrier and non-carrier cattle of the Trio allele. Mean dominant follicle diameter (A) and volume (B). Total follicle volume of all dominant follicles for each genotype (C). Serum FSH concentrations of carriers and non-carriers of the Trio allele (D).

b. Physiology and prevention of pregnancy loss in recipients of in vitro produced embryos (n = 1,600; 28.7% loss from Day 32 to 60) and cloned embryos (~80% loss from Day 32 to 60). The main question is whether pregnancy loss occurs because of inadequate corpus luteum function or inadequate embryo function.

2) Giovanni M. Baez projects:

a. Finished a project in which we evaluated production of a contralateral CL using GnRH on day 5 after AI in 718 lactating dairy cows.
   i. Ovulation near timed AI: Confirmed that ovulation was preferentially on the right ovary in either primiparous (58.3%; 182/312) or multiparous (58.1%; 182/313) cows.
   ii. In pregnant cows (48.0%, 208/433): Accessory contralateral CL underwent luteolysis (67.9%) at a much higher rate than accessory ipsilateral CL (13.0%). There was a similar rate of contralateral CL regression in primiparous (65.8%) and multiparous (70.9%) cows (P = 0.58).
   iii. Timing of CL regression: Early CL regression (days 19-25) occurred more frequently (P = 0.0082) in multiparous (41.0%) than primiparous (15.4%) but later CL regression (d 33-61) occurred more frequently in primiparous (84.6%) than multiparous (59.0%).
   iv. The mechanisms causing contralateral CL regression, particularly around d 40 of pregnancy, are unknown.

b. We hypothesized that incomplete CL regression is reducing fertility during reproductive management protocols in lactating dairy cows. We have completed two projects that evaluated whether we could increase fertility by increasing the dose of prostaglandin F2a or increasing the number of times that we treated with prostaglandin F2a on fertility in lactating dairy cows. We found that either increasing the dose or treating with an extra dose of prostaglandin F2a increased rate of CL regression, particularly in multiparous cows, and increased fertility (Table 2).

Table 2. Effect of treatment with a second PGF on Pregnancy/AI during the Ovsynch (Experiment 2) or Double-Ovsynch (Experiment 1) protocols. Results from Experiments 1 and 2 were combined for the analysis with all cows assigned to the experiments included in the analysis.

Parity	**One PGF % (n/n)**	**Two PGFs % (n/n)**	**Effect of PGF Difference (P Value)**
Primiparous	39.3 (140/356)	40.6 (139/342)	+ 3.31% (P = 0.39)
Multiparous	32.5 (296/910)	36.5 (333/913)	+12.31% (P = 0.043)
P Value	0.04	0.17	
Overall	34.4 (436/1266)	37.6 (471/1251)	+9.45% (P = 0.049)

c. Uterine size matters for fertility in lactating dairy cows.

There are multiple reasons for reduced fertility in lactating dairy cows. We hypothesized that one cause of reduced fertility could be the overall size of the reproductive tract, particularly the uterus, given well-established uterine functions in many aspects of the reproductive process. Thus, the objectives of this study were to evaluate the variability in uterine size in primiparous and multiparous dairy cows and to analyze whether there was an association between uterine size and fertility, particularly within a given parity. Lactating Holstein dairy cows (n = 704) were synchronized to receive timed AI (TAI) on day 81 ± 3 of lactation by using the Double-Ovsynch protocol (GnRH-7d-PGF-3d-GnRH-7d-GnRH-7d-PGF-56h-GnRH-16h-TAI). At the time of the last injection of PGF, uterine diameter was determined at the greater curvature using ultrasound, uterine length was determined by rectal palpation, and uterine volume was calculated from these two measurements. Blood samples were also taken to measure progesterone (P4) in order to assure synchronization of all cows used in the final analysis (n = 616; primiparous, n = 289; multiparous, n = 327). Primiparous cows had greater percentage pregnant/AI (P/AI) compared to multiparous cows (49.8 vs 39.1% at 67 d pregnancy diagnosis, $P = 0.009$). Diameter, length, and volume of the uterus were larger in multiparous than primiparous cows ($P < 0.001$). For multiparous cows, uterine diameter and volume were smaller in cows that became pregnant compared to cows that were not pregnant to the TAI with a similar tendency observed in primiparous cows. Logistic regression and quartile analysis also demonstrated that as uterine volume increased there was decreased P/AI in either primiparous or multiparous cows. Thus, there is a negative association between uterine size and fertility in lactating dairy cows with cows with larger uterus having reduced fertility, particularly for multiparous cows.

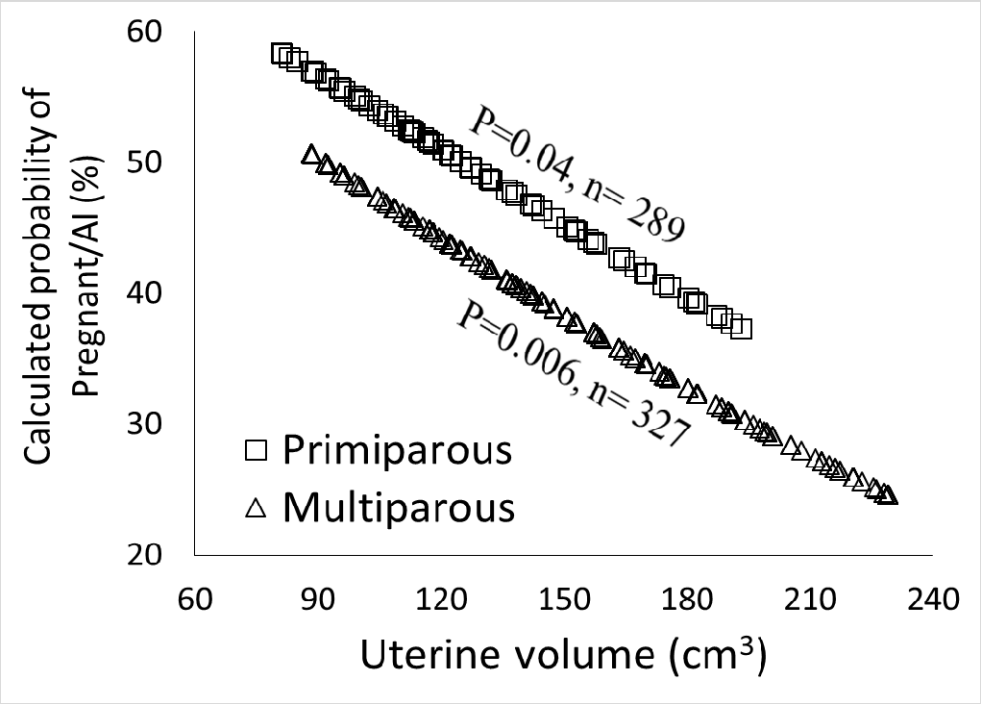

Figure 2. Logistic regressions for association of probability of pregnancy with uterine volume in primiparous and multiparous lactating dairy cows.

3) Mateus Z. Toledo Project:

Experimental objectives were to evaluate the effects of supplementation with rumen-protected methionine (RPM) from 31 ± 2 to 127 ± 2 DIM (61 d after timed AI; TAI) on fertility and embryo development of dairy cows. Holstein cows (n = 309) were housed in a free-stall barn, milked twice daily, fed a basal diet formulated to 16.7 % CP to deliver 2521 g of metabolizable Protein (MP) with 6.93 lysine as % of MP and randomly assigned to once daily top-dressing with either: 1) RPM, 21.2 g of Smartamine M® mixed with 38.8 g of dry distillers grains (2.34 methionine as % of MP) or 2) Control (CON), 60 g of dry distillers grain (1.87 methionine as % of MP). All cows were synchronized using a Double Ovsynch protocol (GnRH-7d-PGF-3d-GnRH-7d-GnRH-7d-PGF-56h-GnRH-16h-TAI) to receive first TAI at 66 ± 3 DIM. Pregnancy diagnosis was done at 28 d (Pregnancy-specific protein B) and at 32 and 61 d (ultrasound) after TAI. Embryo size and amniotic vesicle volume were determined by ultrasound on day 33 of pregnancy. Binomial data were analyzed by parity using Chi-square or Fisher's exact test. Continuous outcomes were analyzed by T-test or Wilcoxon sum-rank test. Only synchronized cows (92.1 %, determined by progesterone concentrations) were used in the final analysis (n = 285). There was no effect of treatment on pregnancies per AI at 28 (65.5 vs. 66.7%; CON vs. RPM), 32 (58.6 vs 61.4%), or 61 (54.4 vs 58.3%) d after TAI. However, pregnancy loss was greater in multiparous cows for CON compared to RPM cows from 28 to 61 d (19.6 vs. 6.1%; P = 0.04) or from 32 to 61 d (8.9 vs. 0.0 %, P = 0.03). However, there was no effect of treatment on pregnancy loss in primiparous cows (28 to 61 d, P = 0.50; 32 to 61 d, P = 0.50). Consistent with data on pregnancy loss, RPM increased embryonic size in multiparous cows (RPM vs. CON; amniotic vesicle volume, 592 vs. 472 mm^3, P = 0.03; embryo abdominal diameter, 11.0 vs. 10.5 mm, P = 0.01; crown-rump length, 5.8 vs. 5.4 mm, P = 0.13), but had little effect on embryonic size in primiparous cows. Thus, top-dressing RPM increased embryo size and pregnancy maintenance in multiparous cows. We hypothesized that incomplete CL regression is reducing fertility during reproductive management protocols in lactating dairy cows. We have completed two projects that evaluated whether we could increase fertility by increasing the dose of prostaglandin F2a or increasing the number of times that we treated with prostaglandin F2a on fertility in lactating dairy cows. We found that either increasing the dose or treating with an extra dose of prostaglandin F2a increased rate of CL regression, particularly in multiparous cows, and increased fertility.

4) Confirmed that circulating AMH concentration was predictive of superovulatory response in lactating dairy cows. (Souza et al., 2015;

Accomplishments

What was accomplished

The long-term goal of this project is to (1) identify intracellular signaling pathways and gene expression regulatory mechanisms that promote oocyte growth and maturation; and (2) identify inter-cellular interactions between somatic cells of the ovary (i.e. theca and granulosa) as well as somatic cells and the oocyte (i.e. granulosa and oocyte) that promote follicular growth and oocyte maturation. This research is focused on understanding dairy cow infertility and providing practical solutions to improve reproductive efficiency for dairy producers. What we accomplished:

1) Determined the physiological responses to cows that were treated with the Double-Ovsynch timed AI protocol. Determined that increased dose of PGF in the Double-Ovsynch protocol increased CL regression and fertility in multiparous dairy cows. Determined that increased dose of GnRH in Double-Ovsynch increased ovulation but did not increase fertility.
2) Determined the effect of acute feed restriction on metabolic and reproductive hormones. Determined that acute feed restriction improved fertilization and embryo quality in cows that had were superovulated using an FSH product with higher LH content. However, using an FSH product with higher LH content compared to a low LH product was inhibitory to fertilization and embryo quality in late lactation cows that were full fed.
3) Determined that deep-horn AI did not improve fertility in dairy cows that had been subjected to superovulation. However, subclinical endometritis decreased percentage of ova that were fertilized and tended to decrease embryo recovery.
4) Determined that BCS loss from calving until 21 d postpartum was related to fertility in lactating dairy cows. Further, cows that had the greatest postpartum loss of weight had a reduction in percentage of embryos that were good quality.

Opportunities for training and professional development

Graduate students performed and analyzed these projects as part of their Ph.D. or M.S. (Paulo Carvahlo) degrees. They were assisted by undergraduates and research interns in many aspects of these projects. Numerous animal techniques, statistical analysis methods, and laboratory procedures were utilized for these projects.

How results have been disseminated to target audience

1) For scientists:
    a. Manuscripts were published (total of 9 reporting results from this project published in 2014) or submitted to peer-reviewed scientific journals for publication.
    b. A total of 11 abstracts were submitted on research from this project and were presented at scientific meetings in 2014.
    a. The P.I. was asked to present at a number of scientific meetings including: Midwest ADSA Ruminant Nutrition Symposium: Amino Acids. March 19, 2014. Wiltbank M. C. Varied effects of multifunctional amino acids on reproduction in lactating dairy cattle. (~150 attendees); Tucker Symposium, Michigan State University. March 24, 2014. Featured lecture. Wiltbank M. C. The tangled web of physiology, management, genetics, and nutrition that underlies reproductive efficiency in lactating dairy cows. (~50 attendees); Cattle Fertility Conference. Galway Ireland. May 20, 2014. Symposium lecture. Wiltbank M. C. Physiological and practical effects of progesterone on reproduction in dairy cattle. (~300 attendees); Society for the Study of Reproduction. Plenary Lecture. July 21, 2014. Wiltbank M. C. Feeding a growing world population: Role of bovine reproductive physiology and reproductive technologies. (~800 scientists in attendance); Brazilian Embryo Transfer Society (SBTE). Natal Brazil. August 15 2014. Moderator and Presenter at Symposium on Improving Embryo Quality. Wiltbank M. C. Nutrition and embryo quality (English). (~250 scientists).
2) For dairy industry professionals:

a. The P.I. presents results at industry meetings including the following veterinary conferences:

Brazilian Embryo Transfer Society (SBTE). Natal Brazil. August 14 2014. Preconference Workshop Presenter. Wiltbank M. C. Improving protocols for timed AI in dairy cattle (everything in Portuguese). (~150 veterinarians or scientists)

Advanced Embryo Transfer Workshop. Seminar 12. September 17, 2014. Wiltbank M. C. Presented topics on advanced embryo transfer from 8:00 AM until 2:30 PM. (40 embryo transfer veterinarians)

American Embryo Transfer Association Meetings. Main Symposium. October 7, 2014. Wiltbank M. C. Optimizing superovulation responses using recent physiological and practical research results. (~220 embryo transfer veterinarians);

Dairy Cattle Reproduction Council Meeting. Salt Lake City, UT. November 14, 2014. Wiltbank M. C. Physiology and management of anovular dairy cattle. (2 presentations of same topic; ~50 producers, industry consultants, veterinarians, and scientists in attendance in each)

b. The BRED (Bovine Reproduction Education and Discussion) workshop for veterinarians continues to be done 2 times per year by P.I., Paul Fricke, and Richard Pursley (limited to 30 veterinarians per workshop). This workshop is an interactive 2 day course that includes small group discussions, data presentations and discussions, and hours of responding to veterinary questions with research data from our trials or the trials of other researchers. Since the BRED workshop began in 2006 we have trained ~350 veterinarians.

c. Two presentations in 2014 at nutritional groups on the effects of nutrition on reproduction.

Plan for next reporting period

Four major studies are occurring on NE1277 at the Wisconsin experiment station in 2015.

Objective 1

1) Determine the effect of supplementation of rumen-protected methionine on reproduction. We are top-dressing methionine (or placebo control) to individual cows on a commercial dairy and evaluating the effects on fertility, pregnancy loss, and embryo size of lactating dairy cows.

2) Determine the timing of regression of an accessory CL in pregnant lactating cows and heifers. We have observed that many accessory CL that are contralateral to the pregnancy will regress between day 19 and 60 of pregnancy. The physiology that underlies this regression may provide insight into the mechanisms that cause pregnancy loss in dairy cows. This project is being combined with an evaluation of gene expression in ipsilateral and contralateral CL during pregnancy.

3) Determine the effect of size of the uterus on fertility in heifers, and primiparous and multiparous lactating dairy cows.

Objective 2

1) We have been working on a high ovulation rate genotype of cattle that has been identified by Dr. Brian Kirkpatrick. Carriers of this allele have an average ovulation rate of 4, whereas non-carrier half-siblings have an ovulation rate of ~1. We are determining the reproductive physiology and molecular pathways that underlie the high ovulation rate due to this allele.
2) The fourth project is to determine the effect of progesterone and CL regression on fertility and pregnancy loss after transfer of in-vitro produced embryos or clones. This project involves over 1500 embryo transfers. Recipients of embryos have been treated or not treated with GnRH on Day 5 and embryos were transferred on Day 7. A second project will use CIDRs to evaluate the number of embryos that are lost due to CL regression as compared to death of the embryo.

Major Changes
N/A